ISOLATION AND CHARACTERIZATION OF A <u>VIBRIO CHOLERAE</u> STRAIN UNABLE TO SECRETE CHOLERA TOXIN, AND CLONING AND CHARACTERIZATION OF GENES FROM <u>VIBRIO CHOLERAE</u> THAT CONFER A CONGO RED DYE BINDING PHENOTYPE ON <u>ESCHERICHIA COLI</u>

1996

COKER

APPROVAL SHEET

Title of Dissertation: "Isolation and Characterization of a *Vibrio cholerae* Strain Unable to Secrete Cholera Toxin, and Cloning and Characterization of Genes from *Vibrio cholerae* that Confer a Congo Red Binding Phenotype on *Escherichia coli*"

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ABSTRACT

Title of Dissertation:

Isolation and Characterization of a *Vibrio cholerae* Strain Unable to Secrete Cholera Toxin, and Cloning and Characterization of Genes from *Vibrio cholerae* that Confer a Congo Red Binding Phenotype on *Escherichia coli.*

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Candidate, Doctor of Philosophy, 1996.

Dissertation directed by:

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Professor and Chairman, Department of Microbiology and Immunology.

Vibrio cholerae is the causative agent of cholera gravis. The main virulence determinant for this organism is the production of cholera toxin (CT), a bipartite toxin enzyme which is responsible for causing the physiological changes in humans that result in massive diarrhea and subsequent fluid loss that is the hallmark of cholera disease. In order for CT to reach the epithelial target cells of the small intestine it

must be synthesized and secreted from the bacterium after infection of the host organism by *V. cholerae*.

The goal of this study was to isolate and characterize genes that encode proteins responsible for the translocation of CT across the outer membrane of *V. cholera*. An El Tor *V. cholerae* strain was isolated by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis that was defective in CT secretion and designated CC9453. CC9453 was found to exhibit a pleiotropic phenotype so far as it was also defective in the secretion of protease, DNAse, and hemolysin enzymes. Attempts to complement the genetic defect responsible for the secretion of these proteins failed. A cloned gene able to complement the CT secretion defect of classical biotype *V. cholerae* strain M14 was unable to complement the defect of CC9453. The nature of the mutation in CC9453 remains unknown.

The second part of this study describes the isolation and characterization of genes from *V. cholerae* that confer a congo red binding phenotype on *Escherichia coli*. Five different alleles were obtained from *V. cholerae* strain U1 that enabled *E. coli* to bind congo red. One of these alleles (*cbp*) was extensively characterized and found to encode a lipoprotein.

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by

Christopher Coker

Dissertation submitted to the

Faculty Department of Microbiology and Immunology

Graduate Program of the

Uniformed Services University of the Health Sciences

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy 1996

ACKNOWLEDGEMENTS

I would like to recognize the following individuals who have made certain contributions towards my graduate education and completion of this dissertation.

Henry Wu - for his encouragement, patience, and invaluable guidance as a dissertation committee member. You will be missed.

Randall Holmes - for his encouragement, understanding, patience, and guidance as my graduate advisor. Also for allowing me to "explore" in the laboratory. Thank-you.

Tony Maurelli - for his encouragement and giving me my start in an intense and educational research setting.

Paul Rick - for his invaluable help as a dissertation committee member and coordinating the completion of this dissertation.

Carl Deiffenbach - for his enthusiasm and contributions towards improving the quality of my research as a dissertation committee member.

and ravings about my latest lab results late night at Sibley.

Jeanine Beckley - for her support, love, and companionship.

Mom and Dad - for always believing in me. I love you.

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INTRODUCTION

Preface

This dissertation is the result of the evolution of two projects. The first part relates efforts to identify the molecular mechanism of cholera toxin (CT) secretion across the membrane of the gram negative bacterium *Vibrio cholerae*. While a CT secretion-negative mutant isolate was obtained, efforts to identify the genetic elements required to restore toxin secretion in this mutant failed. During characterization of the mutant isolate, Overbye, Sandkvist and coworkers had issued two reports describing the preliminary characterization of a multigene complex involved in the secretion of CT from *V. cholerae* (58,65). For these reasons further characterization of the mutant isolate was abandoned in favor of developing a second research project that describes the isolation and characterization of genes from *V. cholerae* that confer a congo red (CR) binding phenotype on *Escherichia coli*.

I. Overview

V. cholerae is a small (0.5 μm x 1.5-3.0 μm) comma shaped gram negative bacterium that is the causal agent of Asiatic cholera (19). Asiatic cholera is characterized by massive fluid and electrolyte loss from the infected individual by means of profuse diarrhea and sometimes

vomiting. The two biotypes of *V. cholerae* O1 that pose the most significant health threats to humans are the Classical and El Tor. Strains of both biotypes that possess the O1 type LPS antigen (serotype O1) are associated with causing Asiatic cholera (19). The El Tor biotype strains are distinguished from the Classical biotype by a) hemolysin production, b) ability to hemagglutinate chicken erythrocytes c) polymyxin B resistance and d) susceptibility to the Mukerjee group IV bacteriophage (19). Genetic loci responsible for hemolysin production, chicken erythrocyte hemagglutination, and polymyxin B resistance have been linked to known markers on the El Tor biotype genome (28). A new El Tor strain of O139 serotype (non-O1 vibrios) emerged in late 1992 and early 1993 and has caused epidemics on the Indian subcontinent (1,34).

Historically there have been seven major recorded pandemics of Asiatic cholera. The seventh pandemic is ongoing currently, although the emergence of *V. cholerae* O139 may signal the beginning of the eighth pandemic (34). An interesting aspect of the seventh pandemic is the introduction of cholera vibrios of the El Tor biotype into Latin and South America where *V. cholerae* had not been a major health threat in the past during this century (52). Also, the emergence of a non-O1 serotype *V. cholerae* strain related to the El Tor biotype has occurred in the midst of the current pandemic. (1,34).

Contaminated food and water are the main sources

for infection by *V. cholerae* (12). Ingested bacteria which survive the acidic environment of the stomach are deposited in the small intestine where they adhere to mucosal surfaces and replicate. During their growth cycle vibrios elaborate CT, the major virulence factor contributing to cholera disease (16). CT is a bipartite toxin consisting of an A (enzymatic) subunit (MW 27,000 Da) and a B (binding) subunit (MW 57,500 Da). Enzymatically active A subunit is formed by the nicking of A into A1 and A2 subunits during holotoxin secretion from *V. cholerae*. (9,19,23,25). Subunit A2 serves to attach the A1 subunit to the B subunit, while the A1 subunit becomes the active enzyme (23,24,25). The B subunit is an oligomer consisting of 5 B subunit polypeptides (MW 11,500 Da) (23,43,57,69). By virtue of the B subunit CT is able to bind to ganglioside GM_1 present on intestinal epithelial cells (81). Only the A1 fragment is delivered to the cytosol of the target cell where it is reduced and released from the A2 subunit (77). The A1 fragment subunit binds to a cellular ADP ribosylation factor-GTP complex to form an activated A1 enzyme (78). The activated enzyme complex is then able to bind NAD^+ and catalyze the transfer of ADP ribose to $G_{s\alpha}$, the positive regulatory protein subunit of adenylate cyclase (10,22,78). GTP is bound by the ADP ribose-modified $G_{s\alpha}$, and the complex activates cellular adenylate cyclase (78). Activated adenylate cyclase catalyses the production of cAMP from ATP (78). Normally GTP bound by unmodified $G_{s\alpha}$ is autohydrolysed (26,78).

The $G_{s\alpha}$-adenylate cyclase complex dissociates and adenylate cyclase activity ceases (26,78). However, due to the ADP ribosylation of $G_{s\alpha}$, cessation of adenylate cyclase activity by hydrolysis of the GTP molecule bound to $G_{s\alpha}$ is inhibited and cAMP accumulates within the toxin treated cell (78). Increased intracellular levels of cAMP may start a cascade of intracellular processes which include release of prostaglandins and arachidonic acid (61,62). The end result is massive fluid secretion into the intestinal lumen of *V. cholerae* infected individuals (17,23). During infection fluid lost from the individual by diarrheal dehydration can be replaced by rehydration salts solutions given either orally or intravenously (51). With prompt rehydration therapy mortality rates due to *V. cholerae* infection can be reduced from >50% to less than 1% (51).

II. Secretion of cholera toxin from *Vibrio cholerae*.

The A and B cholera toxin subunit polypeptides are synthesized in the cytosolic compartment of *V. cholerae* (43,60). After synthesis the precursor polypeptides are translocated through the inner membrane of the bacterium and are presumably processed by signal peptidase I (31,33). The subunits then interact and form a quaternary structure (holotoxin) which is secreted across the outer membrane of the bacterial cell and into the surrounding environment (31,33).

The mechanism of CT secretion across the outer membrane was unknown at the time the project described in this thesis was started and was the major research focus of the thesis. Since then Overbye and coworkers (58) have described a multigene locus involved in the secretion of CT. The purpose of this introduction is to describe the research events leading up to the development of a project that would deduce the mechanism of CT secretion from *V. cholerae*.

A mutant isolate of highly toxigenic classical *V. cholerae* strain 569B Inaba unable to secrete CT was reported by Holmes *et al.* in 1975 (37). This mutant was obtained by NTG mutagenesis and designated M14 (37). Compartmentalization of CT with respect to the periplasm or cytosol in the M14 mutant was not determined in these studies since whole cell sonicate lysates were used in order to determine the amount of cell associated toxin, although subsequent studies demonstrated that CT in *V. cholerae* M14 is localized in the periplasm (31).

In order to study the mechanism of CT secretion a comparison of toxin secretion by *E. coli* and *V. cholerae* was performed by Neill *et al.* (54). Cloned heat labile enterotoxin (LT) genes of *E.coli* were introduced into *V. cholerae* strains 569B and M14. LT is homologous to CT in both structure and function, however, LT is not secreted by *E. coli*. The study by Neill *et al* showed that LT produced by wild type *V. cholerae* 569B was efficiently secreted. When LT genes were transferred to M14, LT was not secreted and remained cell-associated.

Also, Pearson and Mekalanos showed that CT produced in wild-type *E.coli* from cloned CT genes remained cell associated (60). Therefore, *V. cholerae* possesses a mechanism used for toxin secretion that is lacking in *E. coli.*

Hirst and coworkers used a similar approach for studying the secretion of various LT proteins from *V. cholerae* using recombinant plasmids expressing either the A and B subunits, the A subunit only, or the B subunit only (33). *V. cholerae* TRH7000 (an El Tor biotype strain deleted for genes encoding CT) was able to secrete LT holotoxin and the oligomeric B subunit lacking the A subunit, but not the A subunit lacking the B subunit. Cells expressing the A subunit alone exhibited cell-associated subunit A enzymatic activity. *E. coli* cells harboring the recombinant plasmids failed to secrete LT or its subunits. Based on these observations the idea of a "secretory apparatus", present in the outer membrane of *V. cholerae* and able to mediate toxin secretion, was proposed (33).

The localization of the cell-associated toxin in *V. cholerae* was explored in a report by Hirst and Holmgren (31). The antibiotic polymyxin B was used to release proteins from the periplasm of *V. cholerae.* In these studies the intracellular enzyme catechol 2,3-dioxygenase was used as a control for leakage of intracellular proteins across the inner membrane. Log phase cultures of strain TRH7000 harboring an LT encoding plasmid treated with polymyxin B were found to

secrete 70% of total LT present in the periplasm. Pulse chase experiments were performed in order to visualize the transient localization of LT in the periplasm and subsequent release into the supernatants of the cultures over time. Also reported was the observation that the cellular location of CT in M14 was in the periplasm. Thus, M14 was shown to contain a true toxin secretion defect.

A second report by Hirst and Holmgren related that LT B monomers were translocated across the inner membrane of *V. cholerae* and assembled into pentamers in the periplasm (31). Probing for the A subunit by GM1 ELISA revealed that holotoxin was assembled in the periplasm before export across the outer membrane. Assembly of monomeric peptides into oligomers was rapid while secretion of the assembled toxin across the outer membrane was slow in comparison.

The identification of a specific "secretion apparatus" present in *V. cholerae* remained to be resolved. The observations that LT and CT were only secreted by *V. cholerae* and not *E.coli*, the existence of a *V. cholerae* mutant strain unable to secrete toxin, and assembly of holotoxin in the periplasm of *V. cholerae*, suggest that such a secretory apparatus should exist. The goal of this part of the thesis was to identify and characterize genes from *V. cholerae* that encode proteins that comprise the toxin secretory apparatus.

III. Congo red dye binding.

In certain bacterial genera the ability to bind the dye CR correlates with their ability to cause disease in humans. This phenomena has been mostly studied in *Shigella* and *Yersinia spp* (48,48,71,72,75). Generally, those isolates that are able to bind CR are virulent, as defined by a number of *in vivo* and *an vitro* assay systems, while mutant isolates of the same strains unable to bind CR are avirulent in the same assay systems.

In 1977 Payne and Finklestein reported that *V. cholerae* was able to bind CR. A mutant isolate obtained by NTG mutagenesis was unable to bind CR (59). When tested in a chick egg embryo assay for virulence it was found that the mutant isolate unable to bind CR was less virulent than the CR⁻ binding parental strain. Interestingly, the virulence of the CR⁻nonbinding *V. cholerae* isolate was "rescued" when iron was added with the inoculum (59). Further characterization of this mutant isolate was not reported, and this original CR negative mutant of *V. cholerae* has been lost and is no longer available for study (S. Payne, personal communication). In order to better understand the contribution of genes that specify the CR binding phenotype in *V. cholerae* and their potential involvement in virulence in this species, we sought to identify and characterize such genes and their gene products.

IV. Specific aims.

The initial objective of this thesis was to identify and characterize genes from an El Tor *V. cholerae* strain that are responsible for secretion of CT from the periplasm across the outer membrane. The first step required for this analysis was the isolation of one or more mutant strains unable to secrete CT. Genetic complementation by introduction of wild type *V. cholerae* DNA via recombinant plasmids into the mutant isolate would be attempted in order to isolate genes responsible for CT secretion. The genes would then be sequenced and their protein products analyzed in order to try to deduce the role that the proteins played in CT secretion. A mutant isolate, designated CC9453 was obtained, but attempts to complement the defect were unsuccessful. While we were trying to complement the defect, Sandkvist *et al.* informed us that they had succeeded in identifying a gene (*eps*E) that was able to complement the CT secretion defect in M14 (65). Furthermore, they had identified a gene cluster associated with *eps*E that was related to genes involved in the secretion of pullulanase from *Klebsiella* (14). Since attempts to complement CC9453 were unsuccessful and Sandkvist and colleagues had succeeded in identifying a cluster of genes that may be involved in CT secretion, it was decided that a different aspect of *V.* biology be pursued in a new project.

The goal of the second project was to identify and characterize genes from *V. cholerae* El Tor that could confer

a CR binding phenotype on an *E. coli* strain unable to bind CR. One of the approaches was to introduce a gene bank consisting of *V. cholerae* DNA into a non CR binding laboratory strain of *E. coli* in order to isolate allele(s) that conferred CR binding on *E. coli*. The genes would then be characterized by sequencing and their protein products analyzed.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and their genotypes used throughout this study are listed in Table I. Cloning vectors and recombinant plasmids used or generated in this study are listed in Tables II and III.

Media, enzymes, chemicals and radionuclides. Brain heart infusion (BHI)(Difco Laboratories Detroit, MI) and Luria Bertani (LB) (45) medium in broth and agar form were used in the routine culture of bacterial strains. Yeast extract peptone water (YEP) broth (40) was used to grow *V. cholerae* El Tor isolates in order to measure cholera toxin production in the solid phase radioimmunoassay (SPRIA). LB agar containing 0.01% congo red dye (Sigma Chemical Co., St. Louis, MO.) was used in screening for the CR dye-binding phenotype of bacterial isolates. DNAse test agar, skim milk powder, azocasein, hide powder azure, HEPES buffer, and sodium azide were purchased from Sigma. Sheep red blood cells (SRBC) were a kind gift from Dr. Stephanie Vogel, Department of Microbiology and Immunology, USUHS. Antibiotics were incorporated in the above media at the following concentrations when appropriate: ampicillin (Ap) 100 μg/ml, chloramphenicol (Cm) 5 μg/ml, spectinomycin (Sp) 75 μg/ml, tetracycline (Tc) 5 μg/ml, and polymyxin B 2 mg/ml. Globomycin (Sigma) was used at a final concentration of 60 μg/ml and was

TABLE I
Bacterial Strains Used in this Study

Strain	Description	Reference
Vibrio cholerae		
U1	*V. cholerae* El Tor biotype	(56)
GN6300	U1 spontaneous Sp^r derivative	(56)
JN1001	GN6300 *xds-201*::Tn5(pJN8)	(55)
HM100	GN6300 *hly*::Tc^{r}cass (Hly$^-$, Tc^r, Sp^r)	Marcus; unpublished
HM101	HM100 Fusaric acid^r, (Hly$^-$, Tc^s, Sp^r)	This study
CC9452	HM100 CT secretion defective (Hly$^-$, Tc^r, Sp^r)	This study
CC9453	CC9452 FA^r, Hly$^-$, Tc^s, Sp^r	This study
569B	*V. cholerae* classical biotype	(18)
M14	569B CT secretion defective	(37)
Escherichia coli		
HB101	F$^-$ *hsd*S20(r$_{B-}$,m$_{B-}$)*rec*A13 *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *thi*-1 *rps*L20(Sm^r)*xyl*15 λ^- *leu*B6 *mtl*1	(5)
CC118	*ara*D139 Δ(*ara-leu*)7697 Δ*lac*X74 Δ*pho*A20 *gal*E *gal*K *thi* *rps*E(Sp^r) *rpo*B(Sm^r Rif^r Sg^r) *arg*E(Am) *rec*A1	(44)
TKX-1	Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F' *pro*AB *lac*I^{q}ZΔM15 Tn5]pTK; Km^r Tc^r	Stratagene
XL-1Blue	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*[F'*pro*AB *lac*I^{q}ZΔM15Tn10(Tc^r)	Stratagene

DH5α   F⁻ φ80dlacZΔM15 Δ(lacZYA-   Life Tech., Inc.
            argF)U169 deoR recA1 endA1 hsdR17
            ($r_{K.}$,m_{K+}) supE44 λ⁻ thi-1 gyrA96
            relA1

DH5αmcr   DH5α mcrA Δ(mrr-hsdRMS-mcrBC)   Life Tech., Inc.

TABLE II
Plasmids and Cloning Vectors
Used in this Study

Plasmid	Description	Reference
pCVD305	cosmid cloning vector, ColE1 ori; Tc^r	Kaper; unpublished
pBR328	plasmid cloning vector, ColE1 ori; Ap^r, Cm^r, Tc^r	(13)
pACYC184	plasmid cloning vector, p15A ori; Cm^r, Tc^r	(11)
pACYC184*cos*	pACYC184 containing the *cos* site from plasmid pHC79	This study
pHC79	cosmid cloning vector *cos* site is present on a 2.0kb *Bgl*II fragment, pMB1 ori; Ap^r, Tc^r	(36)
pK184	low copy number cloning vector, p15A ori; Km^r	(41)
pMMB66HE	Plasmid cloning vector, ColE1 ori; Ap^r	(21)
pBluescriptSK$^-$	Plasmid cloning vector, ColE1 ori; Ap^r	Stratagene
pBluescriptKS$^+$	Plasmid cloning vector, ColE1 ori; Ap^r	Stratagene
pWSK29	Low copy number plasmid cloning vector, pSC101 ori; Ap^r	(81)
pAMH62	recombinant plasmid carrying *lam*B ColE1 ori; Ap^r	(29)
pGP1-2	recombinant plasmid carrying a thermoinducible T7 RNA polymerase gene	(74)
pMMB384	pMMB66HE carrying the *eps*E allele	(65)

pJBK128   pBR328 recombinant plasmid Kaper; unpublished
           with a w/t hemolysin gene
           cloned from *V. cholerae* 569B

TABLE III
Recombinant Plasmids Used in this Study
(All created in this study)

Plasmid	Description
pCR-1,2,3,7,9,12	recombinant cosmids carrying alleles that confer a congo red dye binding phenotype on *E.coli*; Tc^r
pMR1	pACYC184*cos* containing a 5.1kb *Eco*RI insert from pCR1. CR$^+$ Tc^r
pMR2-1	pBR328 containing a 3.0kb *Eco*RI insert from pCR2. CR$^+$ Ap^r Tc^r
pMR2-2	pBR328 containing a 10.0kb *Eco*RI insert from pCR2. CR$^+$ Ap^r Tc^r
pMR7	pBR328 containing a 4.8kb *Eco*RI insert from pCR7. CR$^+$ Ap^r Tc^r
pMR7-3	pBR328 containing a 2.0kb *Hind*III insert from pMR7. CR$^+$ Ap^r Tc^r
pMR7-5	pBluescriptSK$^-$ containing the 2.0kb *Hind*III insert from pMR7-3. ORF is under *lac* promoter control CR$^+$ Ap^r
pMR7-6	pBluescriptSK$^-$ containing the 2.0kb *Hind*III insert from pMR7-3. ORF is under T7 promoter control CR$^+$ Ap^r
pMR7-9	pMR7-6 containing an *Eco*RI deletion. ORF is under T7 promoter control. CR$^-$ Ap^r
pMR7-10	pBluescriptSK$^-$ containing a 1.4kb *Hind*III fragment from pCR7. ORF is under T7 promoter control. CR$^+$ Ap^r
pMR7-10ΔRI	pMR7-10 containing an *Eco*RI deletion CR$^+$ Ap^r
pMR7-11	pBluescriptSK$^-$ containing a 1.4kb *Hind*III fragment from pCR7. ORF is under *lac* promoter control. CR$^+$ Ap^r
pMR7-11ΔRI	pMR7-11 containing an *Eco*RI deletion CR$^-$ Ap^r

pMR7-13

pBluescriptKS+ containing a *DraI* fragment from pMR7-11. ORF is under *lac* promoter control. CR$^+$ Ap^r

pMR7-14a-c

Three independent isolates of pMR7-13 containing a *NruI-Bst*1107I deletion. CR$^-$ Ap^r

pMR12

pBR328 containing a 2.6kb *Eco*RI fragment from pCR12. CR$^+$ Ap^r Tc^r

pMR12-1

pWSK29 containing the 2.6kb *Eco*RI fragment from pMR12. ORF is under *lac* promoter control. CR^{weak} Ap^r

pMR12-2

pWSK29 containing the 2.6kb *Eco*RI fragment from pMR12. ORF is under T7 promoter control. CR^{weak} Ap^r

a kind gift from Dr. Henry Wu, Department of Microbiology and Immunology, USUHS.

Restriction enzymes, shrimp alkaline phosphatase, and T4 DNA ligase were purchased from Life Technologies, Inc., Gaithersburg, Md., Boehringer Mannheim Biochemicals, Indianapolis, In., and New England Biolabs, Beverly, Mass. Nick translation kits and DNA molecular weight markers, were purchased from Life Technologies, Inc.

SDS, goat anti-rabbit IgG (GARG), N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and agarose for DNA gel electrophoresis were purchased from Sigma. Acrylamide, bis-acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), urea, and ammonium persulfate were purchased from Bio-Rad Laboratories, Richmond, CA. ^{35}S-methionine, Na^{125}I and ^{32}P-dCTP were purchased from Amersham Corp. Arlington Heights, IL. ^{14}C molecular weight protein standards were purchased from Life Technologies, Inc. ^{125}I labeled goat anti rabbit IgG (Sigma) was prepared by Edda Twiddy in our laboratory (6).

Electroporation, transformation, and λ phage infection of bacteria. *V.cholerae* and *E.coli* were electroporated with vector and recombinant plasmids as per Marcus *et al.* (46) using 20% sucrose as the electroporation medium. *E. coli* were transformed using the CaCl$_2$ procedure as per Maniatis *et al.* (45). *E. coli* were also transduced with λ phage Gigapack (Stratagene) extracts packaged with recombinant cosmid DNA as

per Silhavy (68).

Plasmid and U1 chromosomal DNA isolation. Plasmid DNA was purified from cultures of single colony bacterial isolates using the alkaline lysis procedure as described in Maniatis *et al.*(45). An ethidium bromide purification step was added as described by Stemmer (70).

U1 chromosomal DNA was purified using the following procedure developed in our laboratory. U1 was grown overnight in 3.0 ml of BHI broth at 37°C with shaking. One ml of culture was removed to a 1.5 ml eppendorf tube and centrifuged. The bacterial pellet was resuspended in 220 μl of solution I (50mM glucose, 25 mM Tris, 10 mM EDTA; pH 8.0). SDS was added to a final concentration of 1.0% followed by addition of 3.0 μg heat-treated (DNase-inactivated) RNAse and 3.0 μg proteinase K. The mixture was inverted several times and incubated at 56°C for 15 min. One hundred-fifty μg of ethidium bromide was added, followed by tube inversion to mix, and addition of ammonium acetate to 4M and 420 μl phenol-chloroform-isoamyl alcohol (25:24:1). The tube was incubated at 56°C for 2 min followed by inversion 50 times, or, until the solution became emulsified. Bacterial debris was pelleted in a microcentrifuge at maximum speed for 10' and the aqueous phase removed to a new tube. Four-hundred μl of chloroform-isoamyl alcohol (24:1) was added and the tube inverted 10 times followed by brief centrifugation to separate the aqueous and organic phases. The

aqueous phase was removed to a new tube and 0.64 volumes of isopropanol was added. Precipitated DNA was collected by microcentrifugation at maximum speed for 2', washed in 70 % ethanol, and resuspended in 50 μl TE (50 mM Tris pH 8.0, 1mM EDTA). This procedure typically yielded about 2 mg of chromosomal DNA.

*Sau*3A partial digests, sucrose density separation, and collection of the U1 DNA were performed as described in Maniatis *et al.* (45).

Selection of fusaric acid resistant mutants. Fusaric acid resistant mutants of *V. cholerae* HM100 and CC9453 were selected using the protocol of Bochner *et al.* (4). Single colony isolates were purified twice and tested for sensitivity to Tc on LB agar plates containing 5 μg/ml Tc.

NTG mutant selection of HM100 and radial passive immune hemolysis assay. HM100 was mutagenized with NTG as per the protocol of Holmes *et al.* (37). After NTG treatment bacteria were screened in the radial passive immune hemolysis assay essentially as described by Bramucci *et al.* (7) with the following modifications. Bacteria were diluted to yield approximately 10,000 CFU per 15 x 150 mm YEP agar plate. The diluted bacteria were mixed with 7.0 ml molten 1.5% YEP top agar to which 0.5 ml YEP washed SRBC's. Spectinomycin was added to a final concentration of 75 μg/ml, in order to

prevent the possible contamination of the medium by bacteria that may have been present in the SRBC solution. The mixture was poured onto a YEP agar plate and allowed to solidify. A second 7.0 ml 0.8% YEP agar overlay was added and allowed to solidify. Plates were incubated at 37°C overnight. To detect toxin secretion a third 0.8% YEP top agar overlay was added containing 1.0 ml of reconstituted guinea pig complement and 33 μl of G140 goat anti-CTB polyclonal antisera. The top agar was allowed to solidify and plates were incubated right side up for 30 min at 37°C. The presence of CT was visualized by formation of a hemolytic zone (halo) of lysed SRBC surrounding individual colonies. Colonies that were not surrounded by a halo, or appeared to produce smaller halos than the majority of neighboring colonies, were picked with an inoculating needle and streaked for purification on BHI agar containing Sp. Purified colonies were tested in a secondary RPIHA as follows. Single colony isolates were stabbed onto 15 x 100 mm YEP agar plates that were overlayed with 3.0 ml molten 1.5% YEP agar containing 150 μl washed SRBC. Plates were incubated overnight at 37°C. A second 3.0 ml molten 0.8% YEP overlay was added containing 0.3 ml of reconstituted guinea pig complement and 15 μl of G140 antiserum. Plates were incubated right side up at 37°C for 30 min. Colonies that retained the halo-defective phenotype were tested in the solid phase radioimmunoassay for periplasmic and supernatant compartmentalization of CT.

Solid phase radioimmunoassay and preparation of bacterial supernatant and periplasmic extracts. Supernatant and periplasmic extracts of YEP grown *V. cholerae* isolates that failed to produce halos in the RPIHA were subject to the solid phase radioimmunoassay (SPRIA)(73) as follows. Single colony isolates were inoculated into 3.0 ml YEP liquid media and cultured overnight at 37°C with shaking. The O.D.$_{600}$ of the cultures were measured and an amount of the cell culture that was equivalent to an O.D$_{600}$ equal to 1.0 was removed to an eppendorf tube. The bacteria were centrifuged and the supernatant removed to a fresh tube, labelled as the "supernatant extract", and stored on ice until used in the SPRIA. The bacterial pellet was washed once in 0.5 ml fresh media and recentrifuged. The bacterial pellet was resuspended in 1.0 ml fresh YEP containing 2 mg of polymyxin B and allowed to incubate at 37°C for 15 min. The bacteria were pelleted and the supernatant removed. Since polymyxin B allows for the leakage of periplasmic proteins (31) this fraction was labelled the "periplasmic extract" and stored on ice until used in the SPRIA as follows. Briefly, 96 well microtiter plates were overlaid with 25μl of PBS [8.0 g NaCL, 0.2 g KCL, 1.44 g Na$_2$HPO$_4$, 0.24 g KH$_2$PO$_4$ per liter; adjusted to pH 7.4 (45)] containing 50mM G$_{M1}$. The plates were incubated overnight at room temperature in order to allow for adherence of G$_{M1}$ to the microtiter well surface. The liquid was removed from the wells and the plates were air-dried. Plates prepared in this

way were used immediately in the GM1-SPRIA or stored at -70°C until needed. Fifty microliters of supernatant and periplasmic fractions (prepared above) were added to the G_{M1} sensitized wells and serially 2-fold diluted. The plates were incubated for 1 hr at 37°C in order to allow for CT-G_{M1} interactions. The wells were washed five times with PBS containing 1% heat inactivated horse serum (PBS-H). Next, the wells were overlaid with 25μl rabbit anti-CTB polyclonal antisera (lot R10b) diluted 1:8000 in PBS-H and incubated for 1 hr at 37°C to allow for antibody-antigen interactions. The plates were again washed five times in PBS-H, overlaid with 25μl of PBS-H containing ^{125}IGARG (30,000 counts per well), and incubated for 1hr at 37°C. The plates were washed five times with PBS-H and air-dried. The amount of radioactivity bound to each well was determined by gamma scintillation counting and plotted as CPM x 10^3 vs amount of extract present in each well.

DNAse assay. Single colony isolates from streak purified bacteria were inoculated onto DNAse test agar and incubated overnight at 37°C. Zones of clearing surrounding colonies in the opaque medium indicated the presence of DNAse activity and were measured.

Protease assays. Three different assays were used to measure proteolytic activity of bacterial isolates:

Skim milk agar assay - YEP agar plates were supplemented

with YEP broth containing 1.5% skim milk (final skim milk concentration 0.5%). Bacteria were inoculated onto this medium and incubated overnight at 37°C. Zones of clearing in the skim milk agar indicated proteolytic activity and were measured.

The following two assays measured the proteolytic activity of supernatant and periplasmic fractions of bacteria. Single colony isolates were grown overnight in 3.0 ml of YEP broth with shaking at 37°C. The bacterial cultures were diluted 1:100 into 10 ml of fresh YEP in 125 ml erlenmeyer flasks and incubated at 37°C with shaking for 6 hrs. The O.D.$_{600}$ of each culture was adjusted to 2.5. One ml triplicate samples were removed to 1.5 ml eppendorf tubes and subjected to microcentrifugation. The supernatants were removed and kept for future use. The bacterial pellets were washed once in fresh YEP, repelleted, and resuspended in 1.0 ml samples of YEP containing 2 mg/ml polymyxin B. Each suspension was statically incubated at 37°C for 30 min. Bacterial debris was pelleted, and the supernatants were separated and used in the following assays as the periplasmic fractions.

Azocasein assay - a variation of the procedure reported by Plantner (63) was used to determine proteolytic activity of bacterial isolates. Triplicate samples of periplasmic and supernatant fractions (150 μl) of bacterial isolates were added separately to eppendorf tubes containing 250 μl of 2% azocasein and 0.2% sodium azide in 50 mM HEPES buffer (pH 7.5). The tubes were incubated overnight at room temperature.

Extracts containing proteases will digest the casein and release soluble azo dye. Trichloroacetic acid was added to a final concentration of 30% to precipitate undigested azocasein. After microcentrifugation the $O.D._{440}$ of the supernatant, containing solubilized azo dye, from each tube was measured.

Hide powder azure assay - A variation of the hide powder azure assay reported by Rinderknecht et al. (64) was also used to measure proteolytic activity of bacteria. Triplicate samples of supernatant and periplasmic fractions of bacterial isolates (750 μl) were added separately to 13 x 75 mm Falcon snap cap tubes containing 30 mg of hide powder azure suspended in 750 μl of 50 mM HEPES buffer (pH 7.5). Tubes were incubated at 37°C for 30 min and inverted once every 5 min during incubation. Bacterial extracts containing protease activity will digest the hide powder, thereby solubilizing the azure dye. Undigested hide powder was allowed to settle at the end of incubation and the $O.D._{550}$ of 1.0 ml supernatant aliquots, containing solubilized azure, was measured.

Hemolysis assay. YEP agar plates overlaid with 3.0 ml of YEP top agar (agar concentration 0.5% in YEP broth) containing 150 μl of washed SRBC's were inoculated with bacterial isolates and incubated overnight at 37°C. Zones of hemolysis were measured as an indication of hemolytic activity produced by the bacteria.

Congo red binding assay. Bacteria were grown overnight in 3.0 ml LB broth containing appropriate antibiotics. The O.D.$_{600}$ of each culture was adjusted to 1.0. One ml from each of the adjusted cultures was removed to a 1.5 ml eppendorf tube and microcentrifuged. The bacterial pellets were resuspended in 1.0 ml samples of filter sterilized LB containing 50 μg/ml CR dye and statically incubated for 1.0 hr at 37°C. The tubes were inverted once after the first 30 min of incubation. At the end of the incubation period bacteria were collected by centrifugation and washed twice in 0.5 ml of phosphate buffered saline (pH 7.4). After the second wash the bacterial pellets were resuspended in 1.0 ml of 0.5% SDS and incubated at room temperature for a total of 5 min, during which the tubes were vigorously vortexed at one minute intervals. Bacterial debris was pelleted and the O.D$_{500}$ of the supernatants containing CR dye was measured. Triplicate samples were routinely tested.

T7 polymerase assay. The T7 polymerase assay was used to analyze the expression of protein products from the T7 promoter present in recombinant plasmids. The assays were conducted as per Tabor and Richardson (74).

Sequencing. Sequencing of recombinant plasmids was performed following the methods of Sanger *et al.* (66) and instructions accompanying Sequenase sequencing kits from U.S.

Biochemicals.

In situ **DNA hybridizations.** *Eco*RI fragments from recombinant plasmids were gel purified using the FMC purification system (FMC Bioproducts, Rockland, ME). Recovered DNA was labelled with ^{32}P-dCTP following the instructions provided with the nick translation kit from Life Technologies. Unincorporated nucleotides were removed by G-25 quickspin gel filtration columns (Boehringer Mannheim Biochemicals). Labeled DNA was then used immediately in the *in situ* DNA hybridization protocol of Kidd (42) with the following modifications. Hybridization buffer consisted of 1.8 M sodium chloride, 1.2 mM EDTA (pH 8.0), 40 mM sodium pyrophosphate, 0.2% SDS, and 180 mM Tris (pH 8.0). Addition of sodium pyrophosphate in the hybridization buffer precluded nonspecific binding of probe DNA to the dehydrated gel matrix, thus, prehybridization was not required in this protocol.

Cloning procedures. Cosmid and vector plasmid DNAs were digested with restriction enzymes and treated with shrimp alkaline phosphatase (SAP) as per the manufacturers' instructions. After heat inactivation of the SAP and phenol/chloroform extraction (45), the DNA samples were ethanol precipitated and resuspended in sterile deionized H_2O. Insert DNA, ligase buffer, and ligase (1.0 unit for routine ligations and 5.0 units for shotgun cloning and bank

construction) were added to the digested vectors. Ligation was allowed to proceed for three hours at room temperature (for inserts with 'sticky' ends) or overnight at 16°C (for blunt end ligations and bank construction). The ligated DNA samples were stored at 4°C.

RESULTS

Part I - Construction of a Toxin Secretion Mutant of *Vibrio cholerae* El Tor Biotype Strain U1

Isolation of a toxin secretion-negative *Vibrio cholerae* strain. HM100 was used as the target for NTG mutagenesis in order to isolate a toxin secretion defective *V. cholerae* mutant. HM100 was treated with NTG, diluted, and screened for the potential toxin secretion mutant phenotype in the RPIHA. Bacterial killing by NTG was used as an indicator of the potency of the stock NTG solution used in the mutagenesis procedure. Over 90% of NTG treated bacteria were killed.

Approximately 11,000 CFU of NTG treated bacteria were screened for decreased production of extracellular CT by the RPIHA procedure. Ninety-three isolates were picked that demonstrated smaller halo formation than the majority of colonies on the plate. These isolates were picked and purified by single colony isolation on BHI agar containing spectinomycin. One colony from each purification was picked and rescreened in a second RPIHA using HM100 as a positive control. In retrospect, several colonies from each purification should have been picked and rescreened. Wild type bacteria may have inadvertently been present in the original inoculum used for purification. Of the ninety-three isolates

rescreened, forty-one retained the inability to produce a wild type halo. Again, each of the forty-one isolates was picked and purified on BHI agar containing spectinomycin. Single colony isolates from these purifications were screened in the GM1-SPRIA. After overnight culture of the bacterial isolates in YEP broth containing Sp, supernatant and polymyxin B (periplasmic) extracts were obtained and tested in the GM1-SPRIA for CT activity. One isolate, designated CC9452, exhibited a smaller halo on RPIHA plates compared to HM100, and was found to have almost all of the detectable CT present in the periplasmic fraction of the extracts (Figure 1). The remaining isolates either failed to produce CT or produced low levels of CT that were secreted normally into the culture supernatant.

Construction of HM101 and CC9453. Fusaric acid (FA) resistant and tetracycline-sensitive derivatives of HM100 and CC9452 were isolated in the anticipation that Tc^r plasmids might be introduced into these strains at a future time. Also, the Tc^r cosmid vector pCVD305 was being considered for possible use in future experiments directed towards complementation of the mutant toxin secretion phenotype exhibited by CC9452.

Samples containing 100 μl from overnight cultures of HM100 and CC9452 were inoculated separately onto FA agar plates. From each plate three colonies were picked and

Figure 1 Legend

Cholera toxin secretion exhibited by HM100 and CC9452.
Bacteria were grown overnight in YEP broth at 37°C with shaking. Supernatant and periplasmic extracts cultures were assayed for the presence of CT in the GM1-SPRIA. Extracts were 2-fold serially diluted onto GM-1 coated microtiter plates, followed by washing and binding of polyclonal rabbit anti-B immune sera. GM-1-CT-antibody complexes were washed and overlaid with goat anti-rabbit IgG labelled with ^{125}I. After washing, the remaining ^{125}I in each microtiter well was counted in a scintillation counter. Counts per minute (cpm) remaining in the microtiter well (which is an indirect measure of the quantity of CT present in each well) are plotted against the corresponding amount of extract present in the well. The designations "S" and "P" following the strain name refer to supernatant and periplasmic samples of the bacterial culture, respectively. This figure shows a representative graph of several assays.
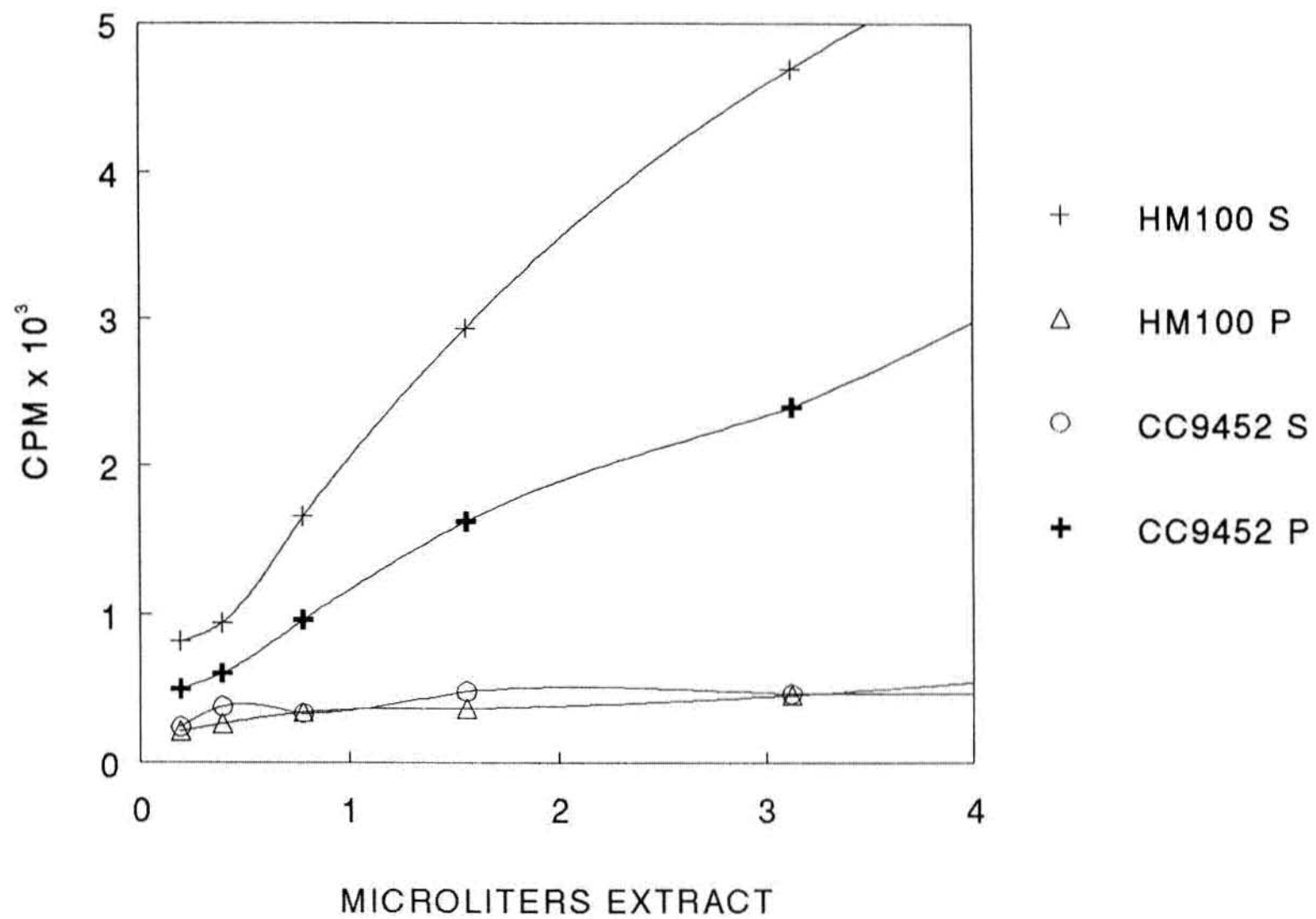
purified by two successive single colony isolations on FA agar plates. Single colony isolates from the purification were picked to LB agar plates containing Tc. Isolates unable to grow on the Tc containing media were designated HM101 and CC9453 respectively. HM101 and CC9453 were then analyzed for their CT secretion phenotypes.

HM101 and CC9453 were subcultured to fresh YEP, incubated for four hours, and samples were collected for analysis of CT in supernatant and periplasmic fractions. Initially, HM101 transiently accumulated some CT in the periplasm, however, all of the CT was released into the supernatant during late log phase. (Figure 2A). In contrast, throughout early log phase CC9453 accumulated most of the CT in the periplasm, and during late log and stationary phase less than half of the total CT was released into the culture supernatant (Figure 2B).

Overnight cultures of HM101 and CC9453 were also tested for CT in the supernatant and periplasmic fractions by GM1-SPRIA. HM101 released approximately 97% of total CT into the supernatant while CC9453 released only 20% of total toxin into the supernatant (Figure 2C). In repeated experiments there was some variability in the amount of toxin released into the supernatant in overnight cultures of CC9453 from approximately 20% to 50%.

Figure 2 Legend

Cholera toxin secretion profiles of HM101 and CC9453 throughout the growth curve. Bacteria were grown overnight in YEP medium and subcultured to fresh medium. The O.D.$_{600}$ of the cultures was monitored as well as CT expression by GM1-SPRIA at one hour intervals.

A. CT secretion and growth curve of HM101.

B. CT secretion and growth curve of CC9453.

C. Cholera toxin secretion of HM101 and CC9453 after overnight growth. Bacteria were grown overnight in YEP broth at 37°C with shaking. Cholera toxin was measured by GM1-SPRIA as described previously. The designations "S" and "P" following the strain name refer to supernatant and periplasmic extracts of the bacterial cultures, respectively.

O.D.600

TIME (HRS)

TOXIN (ng/ml)/O.D.600

CC9453 S   CC9453 P   O.D.600
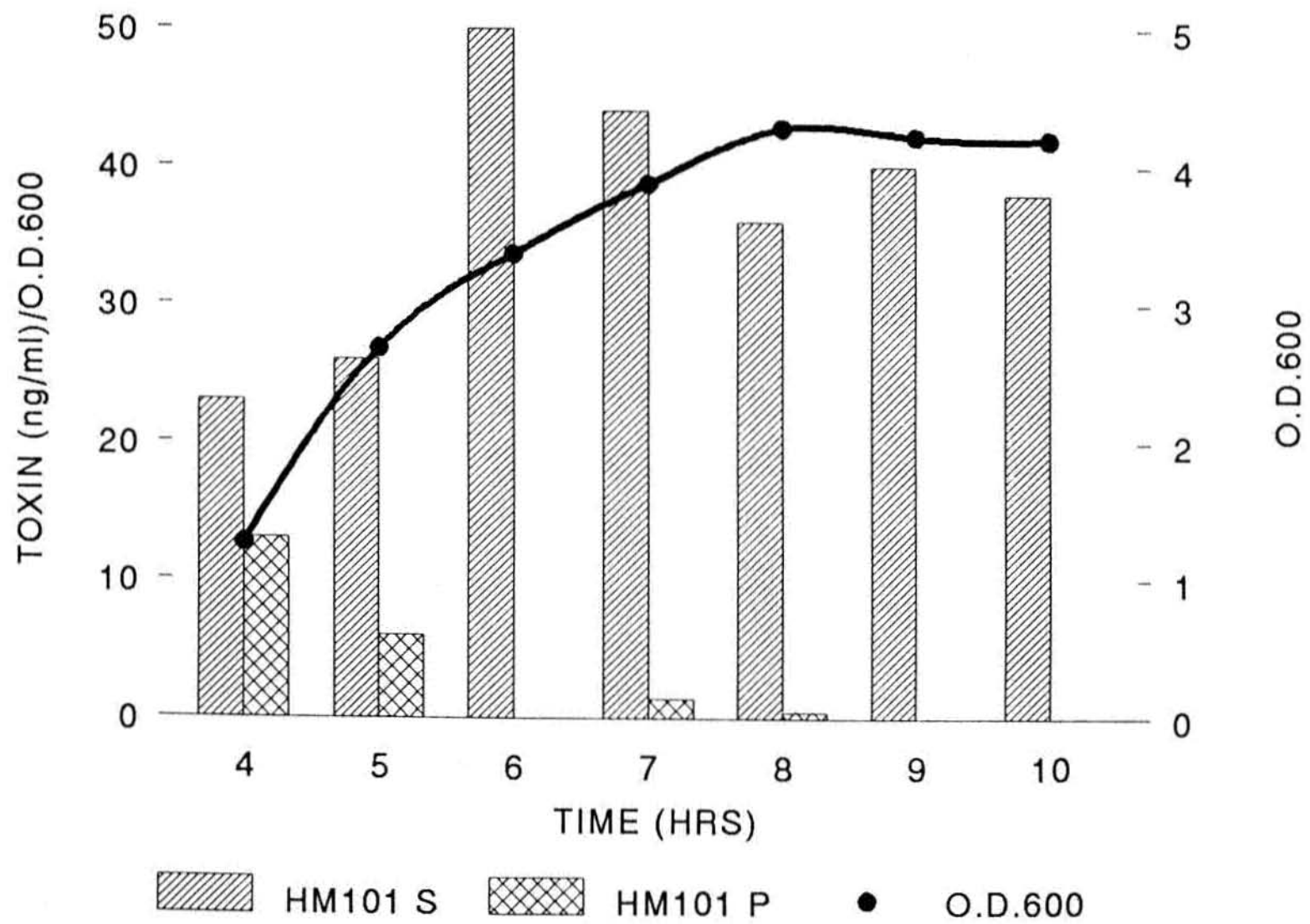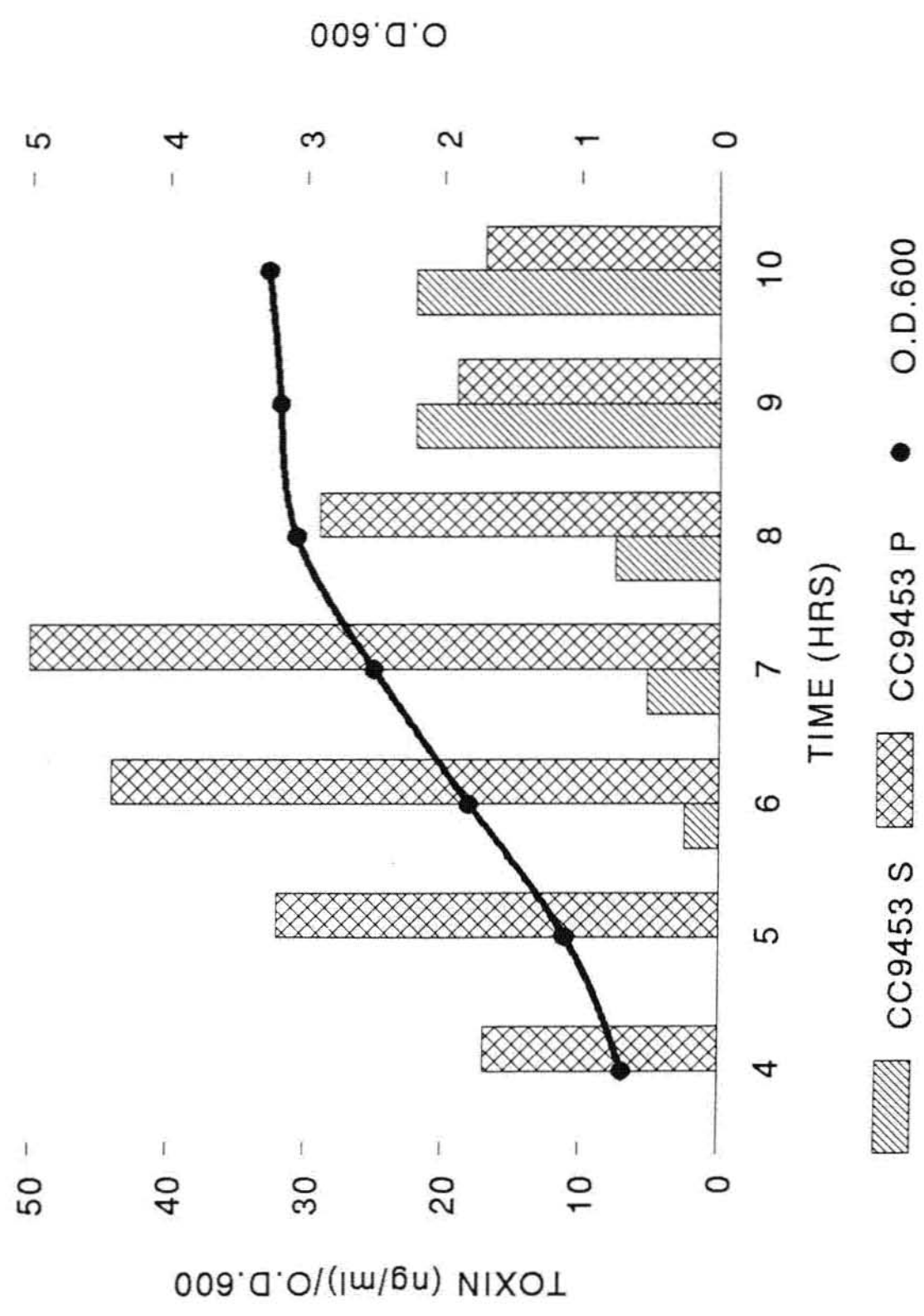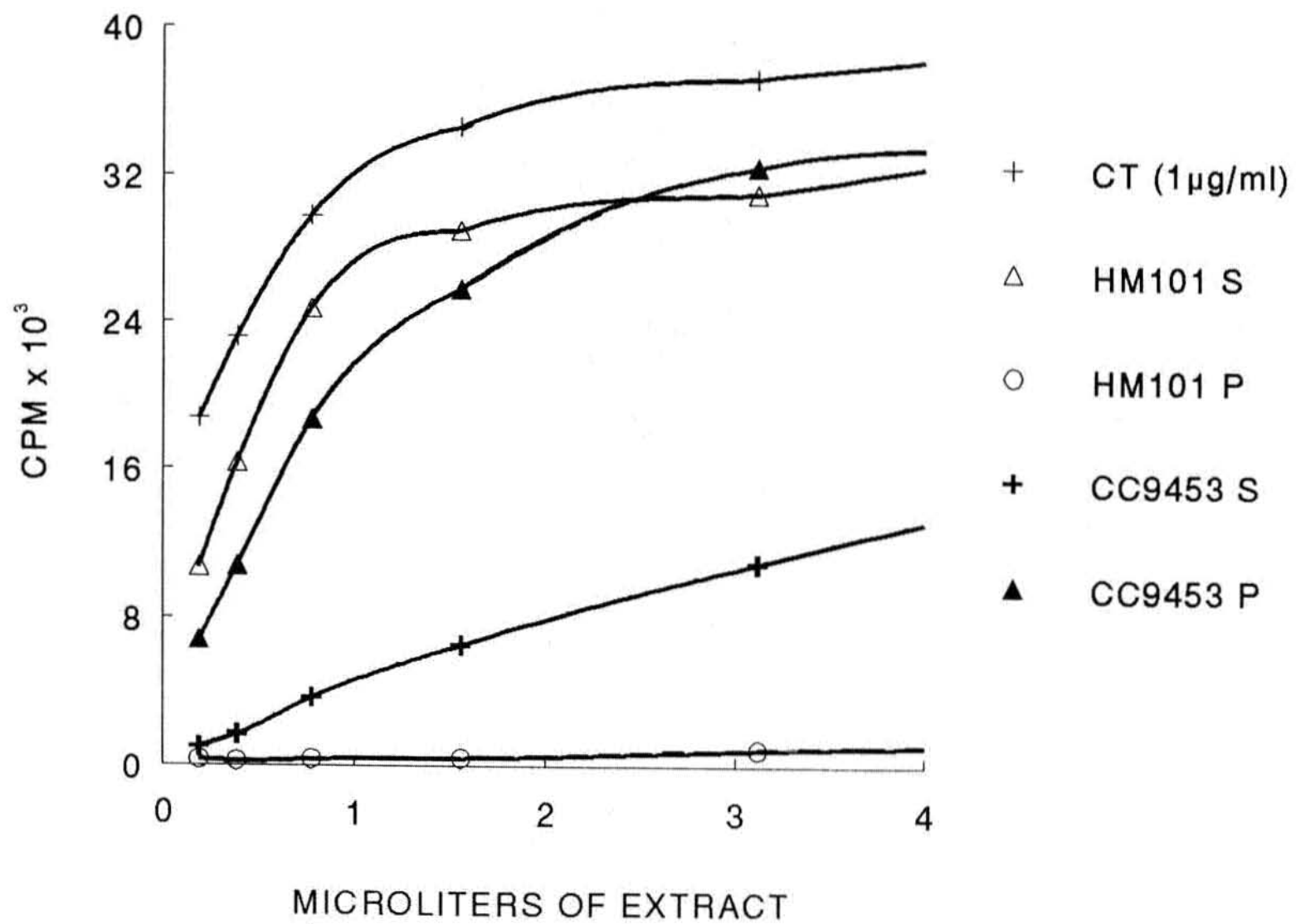
Extracellular Secretion of DNAse, Protease, and Hemolysin from CC9453. Since CT secretion is altered in CC9453, we tested whether secretion of other extracellular proteins was also affected. CC9453, HM101, and a DNase negative mutant of *V. cholerae*, JN1001, were streaked onto DNAse test agar plates. CC9453 exhibited a zone of DNAse activity that was less pronounced than the zone produced from HM101 (Figure 3), and *V. cholerae* JN1001 served as the negative control.

CC9453, HM101, and *E.coli* HB101 were tested in three different assays for protease activity. On YEP skim milk agar plates CC9453 exhibited smaller zones of clearing than HM101, and *E. coli* HB101 exhibited no zone of clearing (Figure 4A). This assay only measures extracellular protease activity. Other assays were used to determine if periplasmic protease activity was altered in CC9453.

Supernatant and polymyxin B (periplasmic) extracts of the above bacteria were inoculated into azocasein assay medium containing azide and incubated overnight. The amount of digested azocasein was measured spectrophotometrically. Supernatants from CC9453 were reduced in their ability to digest the azocasein by approximately 70% in comparison to HM101 (Figure 4B). There was little to no protease activity observed in the periplasmic fractions.

Supernatants and periplasmic extracts from the above strains were also inoculated into a suspension of hide powder

Figure 3 Legend

DNAse secretion by CC9453. Bacteria were picked from single colony isolates, inoculated onto DNAse test agar plates, and incubated at 37°C overnight. Zones of clearing in the agar represent DNAse activity. CC9453 exhibits a smaller zone of clearing than HM101, indicating that DNAse is not secreted from this strain as efficiently as from the parent strain. The negative control represented by *V. cholerae* JN1001 does not have a zone of clearing.
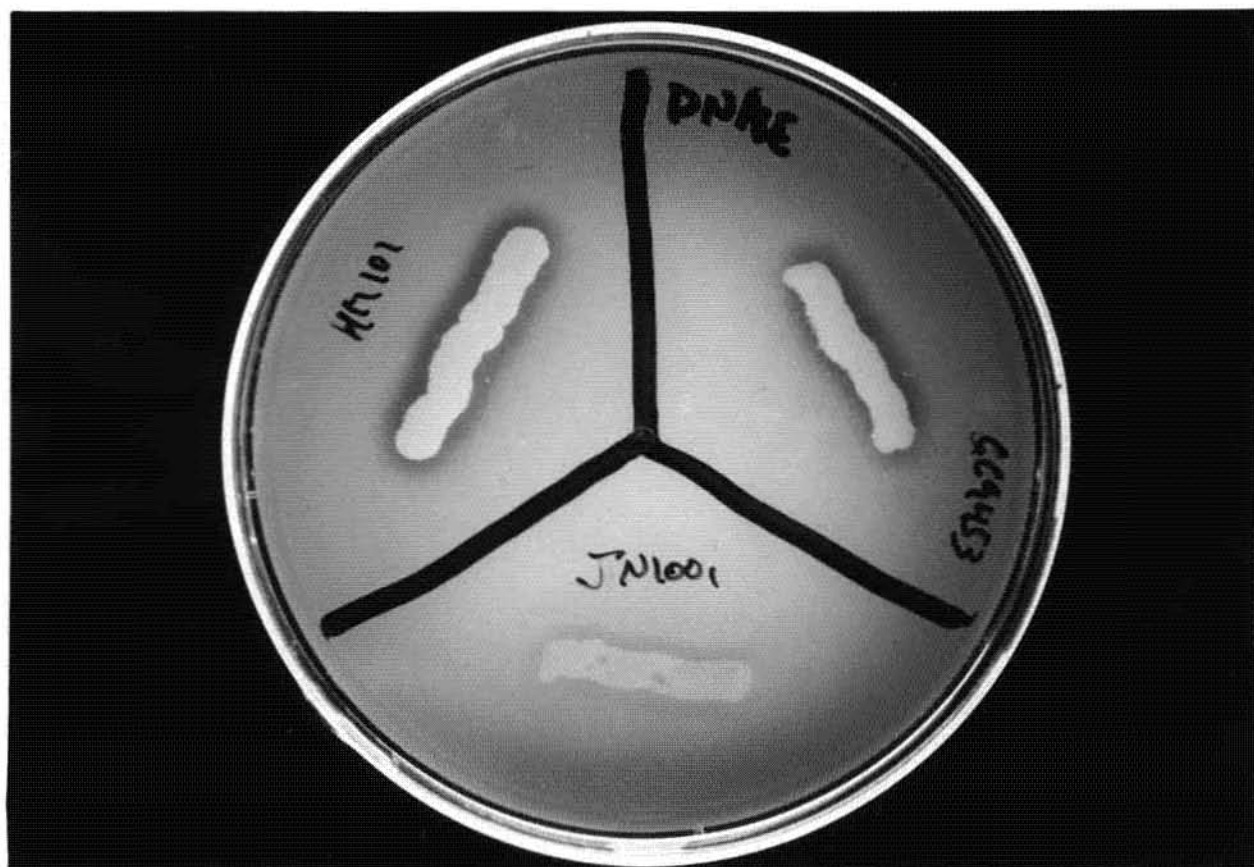
Figure 4 Legend

A. Protease secretion of CC9453 as measured on skim milk agar plates. Bacteria were picked from single colony isolates, inoculated onto skim milk agar plates, and incubated at 37°C overnight. Zones of clearing in the agar represent protease activity. CC9453 exhibits a smaller zone of clearing than HM101, indicating that protease is produced in smaller amounts or is not secreted by this strain as efficiently as from the parent strain. The negative control represented by *E. coli* HB101 does not have a zone of clearing.

B. Protease secretion of CC9453 as measured by the azocasein protease assay. Periplasmic and supernatant fractions of bacterial cultures were incubated overnight in the presence of azocasein. After trichloracetic acid precipitation of undigested azocasein, the O.D.$_{440}$ of the supernatants was measured.

C. Protease secretion of CC9453 as measured by the hide powder azure assay. Periplasmic and supernatant fractions of bacterial cultures were incubated for 30 mins in the presence of hide powder azure. After settling of the azure particles the O.D.$_{550}$ of 1.0 ml aliquots of the supernatant was measured.
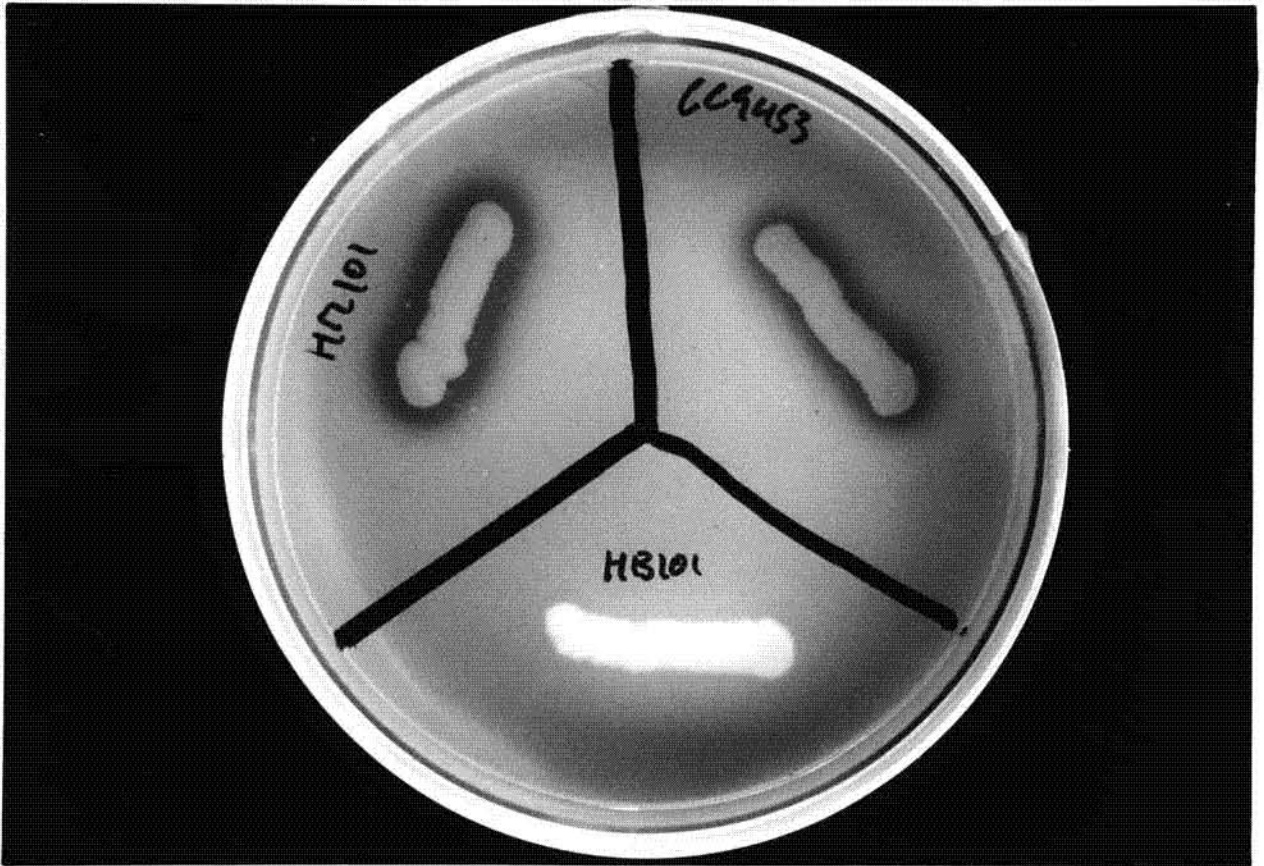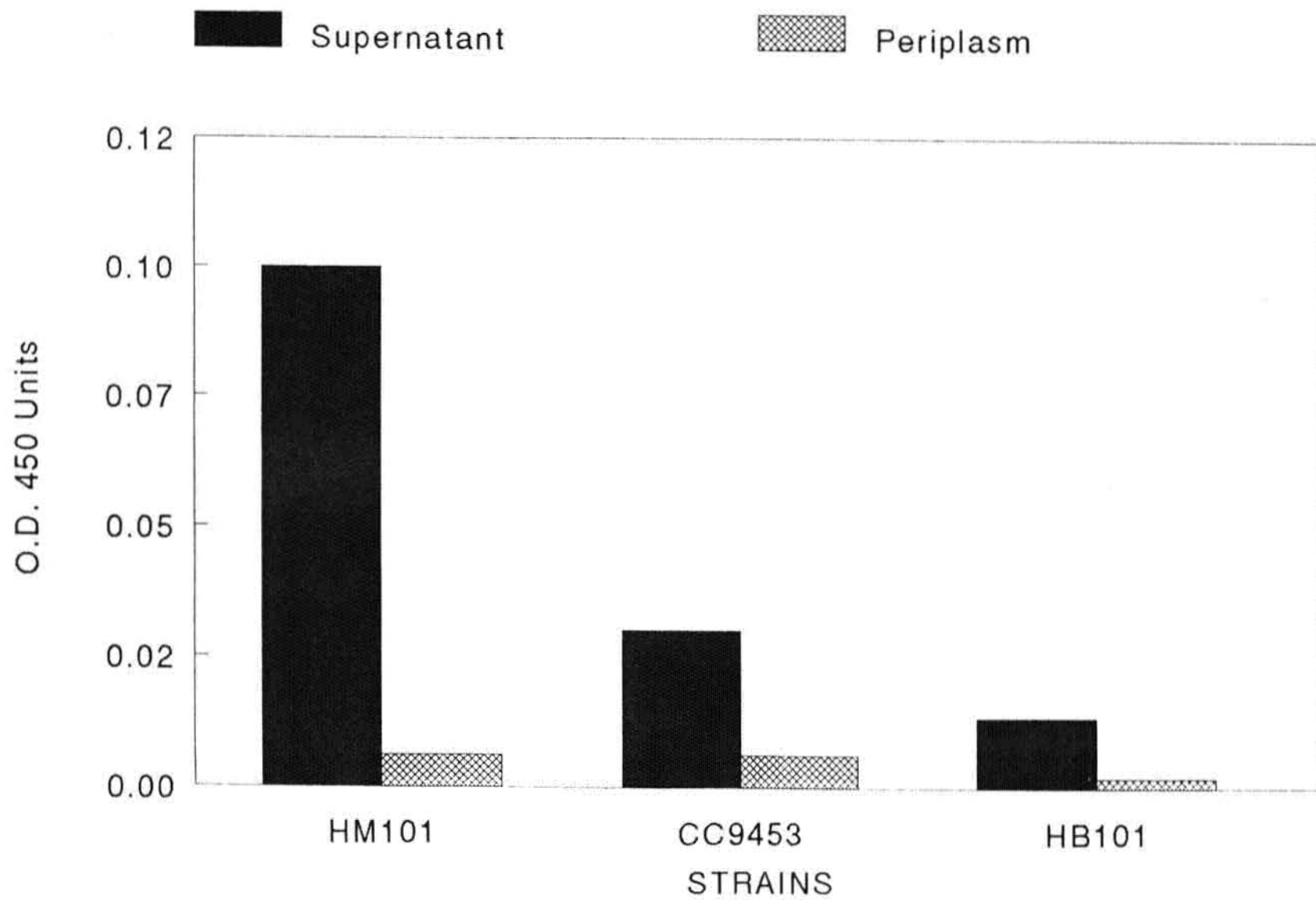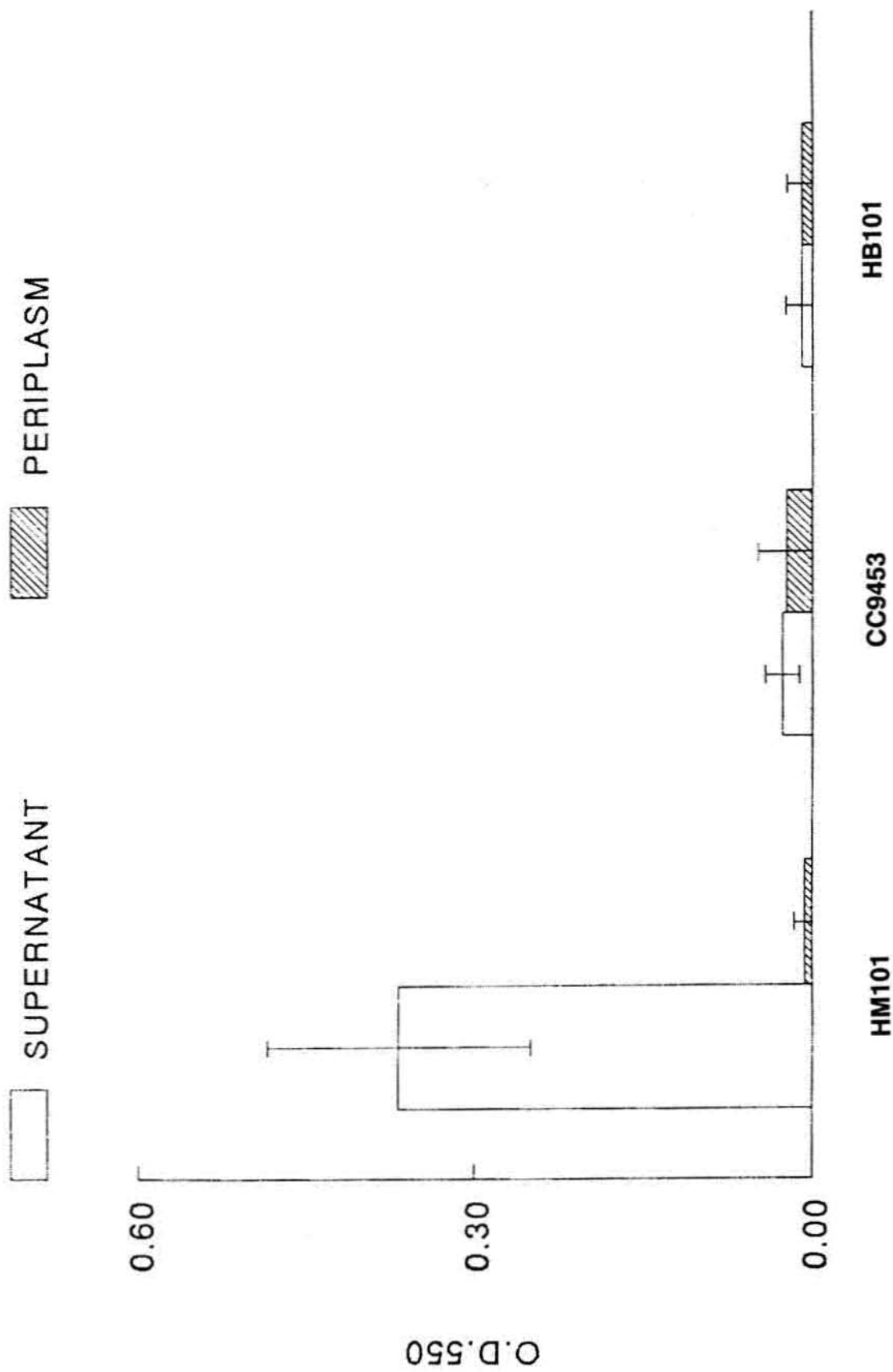
azure. After 30 min incubation the amount of solubilized azure was measured spectrophotometrically. Supernatant extracts from CC9453 were reduced in their ability to digest the hide powder azure by approximately 90% (Figure 4C). Again, no protease activity was observed in the periplasmic fractions or from the negative control strain HB101.

In a number of bacterial genera able to secrete proteases, processing and activation of the protease proteins occurs during their secretion from the periplasmic compartment of the bacteria to the external milieu (80). If such a process occurs in *V .cholerae*, protease activity might not be detected in periplasmic fractions from the above bacterial cultures.

Since HM101 and CC9453 do not produce hemolysin, both HM101 and CC9453 were transformed with plasmid pJBK128. pJBK128 contains an intact *V. cholerae* hemolysin gene (Jim Kaper, personal communication). The hemolysin producing ancestral strain U1 was transformed with pBluescript to serve as an ampicillin resistant (Ap^r) positive control strain. When inoculated on sheep red blood cell agar overlay plates containing Ap U1/pBluescript, HM101/pJBK128, and CC9453/pJBK128 produced hemolytic zones of 3, 4, and 2 mm, respectively. The hemolysin gene product in HM101/pJBK128 and CC9453/pJBK128 may have been produced at a higher level than in strain U1 containing pBluescript, due to the multicopy nature of the pJBK128 plasmid vector, resulting in production of a larger halo by HM101/pJBK128 than by the ancestral strain

U1. In this light the inability of the CC9453 transformant to produce as large a halo as the ancestral strains, together with the results obtained from the protease and DNAse assays above, appears to be a consequence of the disruption of the general protein secretion pathway of *V. cholerae*.

Attempts to complement the toxin secretion defect of CC9453. Initially, cosmid vector pCVD305, a cosmid vector derivative of pACYC184 (pACYC184*cos*) constructed in our laboratory by ligating the *Bgl*II fragment of vector pHC79 (which contains the λ *cos* site) to *Bcl*I digested pACYC184, and the plasmid vector pMMB66HE were used in attempts to construct chromosomal banks of U1 DNA for complementation experiments in CC9453. CC9453 harboring pAMH62 (a plasmid carrying the *lamB* gene from *E. coli*) was unable to be transduced by λ phage. In addition, CC9453 was poorly transformed by electroporation using the plasmid-derived DNA banks. Restriction of the *E. coli*-derived plasmid vectors by *V. cholerae* may have occurred during the transformation event.

In order to circumvent the possibility that a restriction barrier in *V. cholerae* prevented transformation with *E. coli* derived DNA, both pBluescriptKS (pBSKS) and pACYC184*cos* were transformed into and purified from CC9453. Plasmid vectors obtained in this manner should not be subject to restriction upon reintroduction into *V. cholerae* CC9453. The purified vectors were digested with *Bam*HI, ligated to 8-12

kb purified *Sau*3A partially digested U1 chromosomal DNA. The ligation mixtures were transformed into *E. coli* TKX-1, in a control experiment, and electroporated into CC9453. When the plasmid bank was transformed into *E.coli* TKX-1, 75% of the resulting colonies were unable to metabolize the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) under inducing conditions indicating that these transformants contained recombinant plasmids carrying *V. cholerae* DNA.

Approximately 154,000 CC9453 colonies transformed with the pBSKS-derived bank were selected and screened in a primary RPIHA. According to the control transformation in *E. coli*, 115,000 of the 154,000 CC9453 transformants screened above were expected to contain insert DNA. Since the plasmid bank was constructed using *V.cholerae* insert DNA with a minimum size of eight kb, approximately 230 complete U1 chromosomes were screened in these assays, assuming a chromosome size of 4.0×10^6 base pairs (Brenda Talley, personal communication) and random ligation of the insert fragments.

Colonies resulting from the transformation that appeared to make larger halos than CC9453 in the RPIHA were subjected to a second round of RPIHA screening, and twelve putative secretion-positive isolates were selected for screening in the GM1-SPRIA. None of the twelve were confirmed as secretion-positive strains, although they retained the Ap^r marker from the vector plasmid.

Because pBSKS is a high copy number plasmid vector and

we were concerned that overexpression of proteins involved in the general protein secretion pathway may be lethal to *V. cholerae*, a low copy number vector was used to make a chromosomal bank for additional screening attempts. For this purpose we selected pACYC184*cos*. First, a bank made in this vector with large insert fragments was packaged into λ, but efforts to transduce these recombinant cosmids into CC9453/pAMH62 were not successful. Expression of the *lamB* gene product, which is encoded by pAMH62 and serves as the λ receptor, is apparently not effective in *V. cholerae* CC9453. Next, another plasmid bank created by ligating low molecular fragments (8-12kb) of the U1 chromosome into pACYC184*cos* was electroporated into CC9453, and approximately 52,000 transformants were selected for the chloramphenicol resistance marker of pACYC184*cos* and simultaneously screened for CT secretion on RPIHA plates. Colonies that exhibited larger halos than the CC9453 parental strain were selected for a second round of screening in the RPIHA. From the second screening, fourteen putative secretion-positive candidates were grown in overnight cultures and tested in the GM1-SPRIA. Again, none of the fourteen isolates was confirmed to be secretion-positive. In a control experiment testing of 100 chloramphenicol resistant transformants from this bank yielded 95 Tc' isolates, indicating that 95% of the transformants contained *V. cholerae* insert DNA. By extrapolation approximately 49,000 of the 52,000 isolates tested from the

pACYC184*cos* bank should have contained insert DNA representing the equivalent of approximately 98 U1 chromosomes. Use of both high and low copy number vectors (pBSKS and pACYC184*cos* respectively) in the generation of plasmid banks containing U1 DNA did not yield a cloned fragment that could complement the secretion defect in CC9453. The nature of the toxin secretion defect exhibited by CC9453 remains unknown. The possibility that CC9453 contains multiple mutations due to use of NTG cannot be ruled out.

The defective toxin secretion phenotype exhibited by C9453 is not restored by *eps*E. The *eps*E allele derived from *V.cholerae* strain 569B encodes a cytoplasmic protein that is expressed in *V. cholerae*. This allele is able to restore the toxin secretion defect mutation in *V. cholerae* M14 (65), a classical biotype strain derived from 569B by NTG mutagenesis (37). When *eps*E in pMMB366HE (kindly provided by M. Bagdasarian) was transformed into CC9453 it did not restore toxin secretion either under inducing or noninducing conditions (the *eps*E gene is under control of the *lac* promoter present in this plasmid), as determined by GM1-SPRIA of supernatant and periplasmic extracts (Figures 5A and 5B).

Figure 5 Legend

*eps*E does not complement the toxin secretion defect of CC9453. CC9453 harboring the *eps*E allele was tested for CT secretion in the GM1-SPRIA (as described previously) after incubation in the presence (A) or absence (B) of 4 mM IPTG (inducing and noninducing conditions for expression of EpsE from the *lac* promoter present in the plasmid vector).

CPM x 10^3 vs Microliters of Extract

Legend:
+ HM101/PMMB66HE SUPERNATANT
△ HM101/PMMB66HE PERIPLASM
▽ CC9453 /pMMB384 SUPERNATANT
◇ CC9453 /pMMB384 PERIPLASM
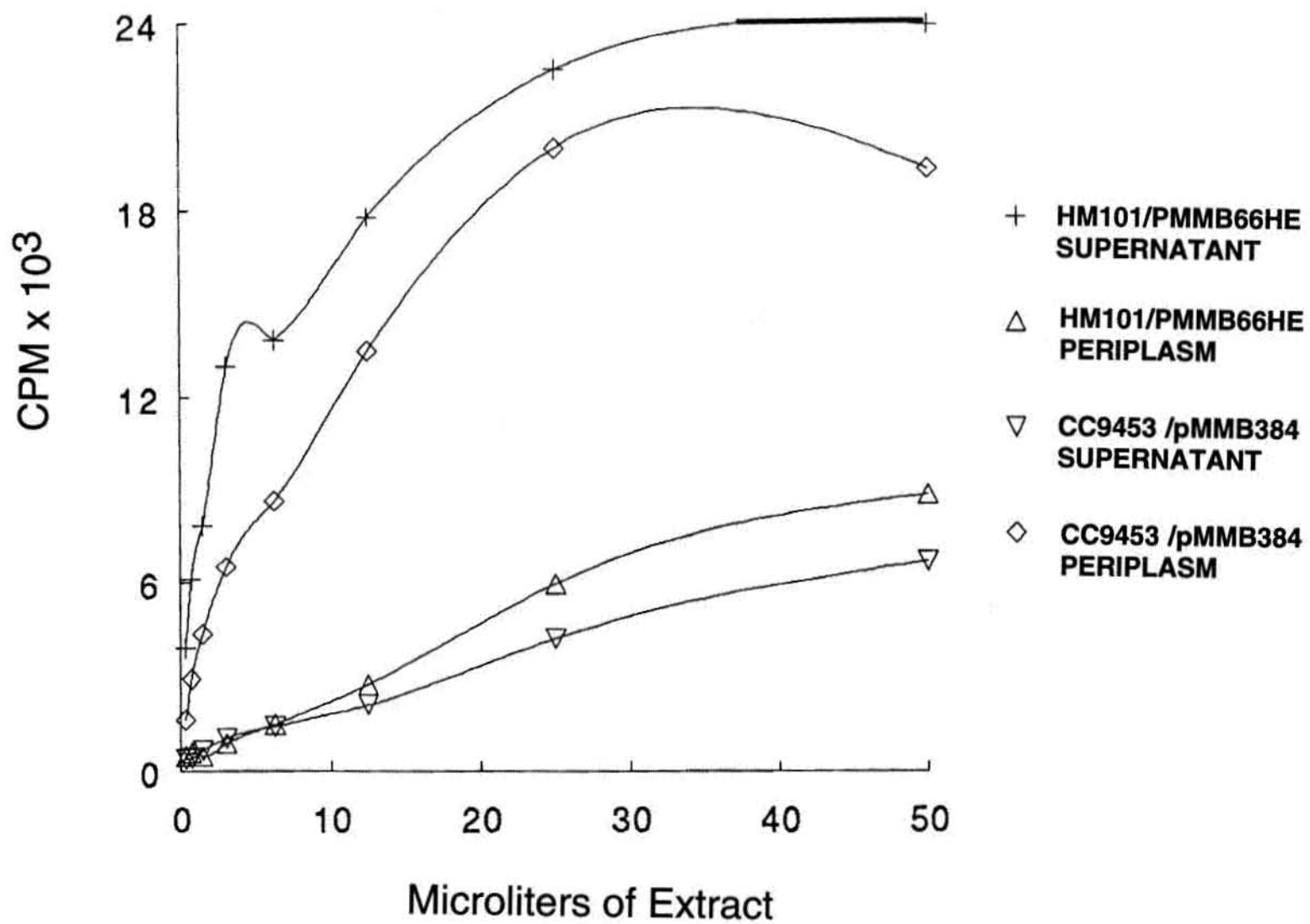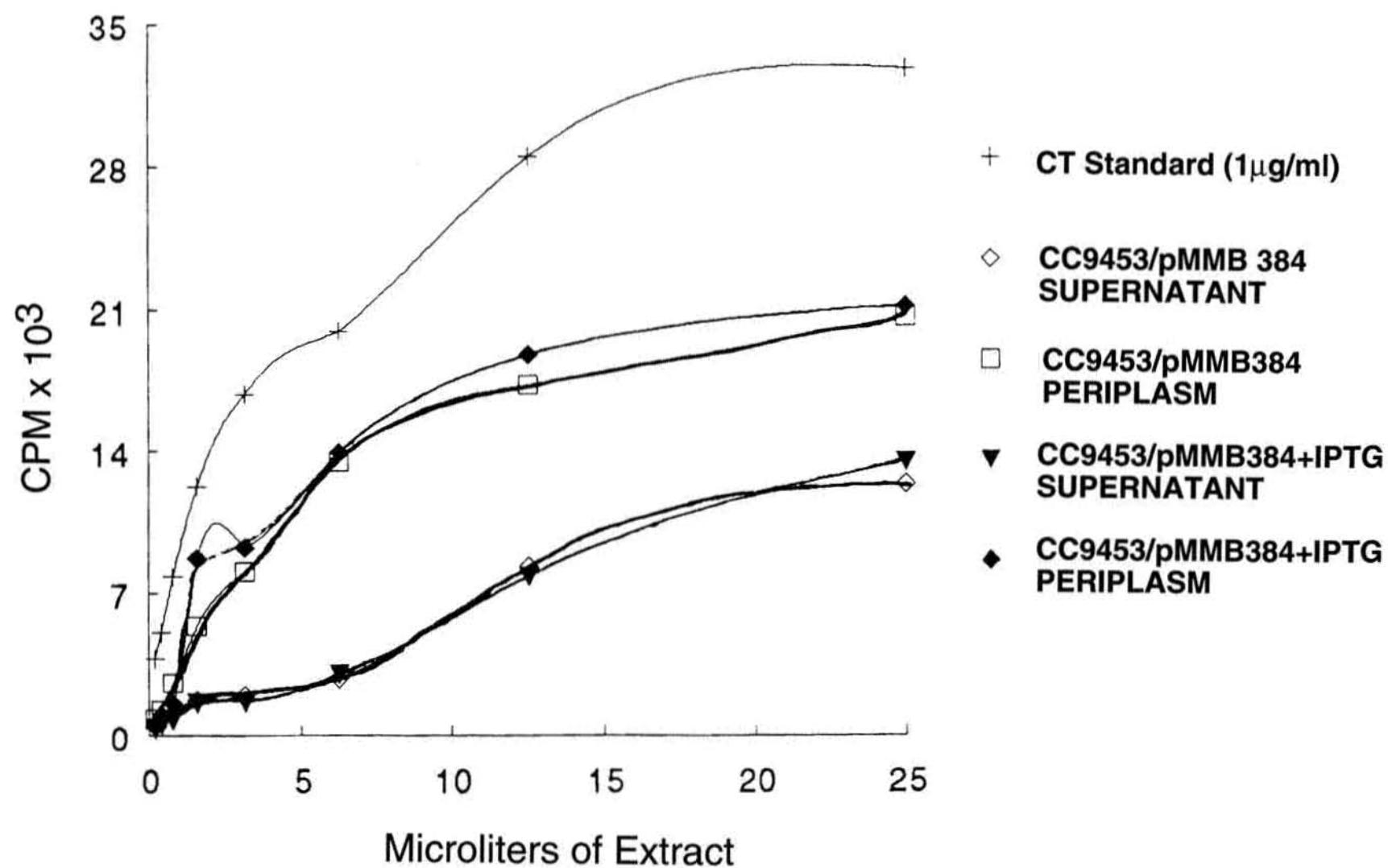
Part II - Characterization of Genes from *Vibrio*
**   *cholerae* that Confer a Congo Red Binding Phenotype**
**   on *Escherichia coli*.**

Screening of a U1 cosmid bank in *E. coli* strain DH5α*mcr* for ability to confer a congo red (CR⁺) binding phenotype. *V. cholerae* U1 chromosomal DNA was partially digested with *Sau*3A and size fractionated by sucrose density centrifugation. A cosmid bank consisting of high molecular weight *Sau*3A chromosomal DNA fractions was ligated to the cosmid vector pCVD305, packaged into λ phage particles. In order to reduce restriction of *V. cholerae* DNA, the packaged recombinant cosmids were transduced into the restriction deficient *E. coli* strain DH5α*mcr*. Tc^r colonies were selected on LB agar containing 0.01% CR dye and 5μg/ml Tc. Twelve of 508 resulting colonies (~2.4%) exhibited the ability to bind CR dye to varying degrees as judged by eye. These twelve CR⁺ colonies were picked and repurified on CR agar containing 5μg/ml Tc. Eight isolates retained the ability to bind CR. The eight isolates were designated pCR1, 2, 3, 4, 5, 7, 9, and 12 and characterized further.

Characterization of the CR+ cosmid DNA. Two of the eight original isolates (pCR4 and pCR5) were found to be unstable and the corresponding recombinant cosmids were lost during the course of this work. The nature of the instability

of these cosmids remains unknown. One of the cosmid isolates (pCR12) was found to revert readily to a CR⁻ phenotype on passage.

Cosmid DNA was purified from cosmid isolates pCR 1, 2, 3, 4, 5, 7, 9, and 12, digested with *Eco*RI, and analyzed by agarose gel electrophoresis (Figure 6). The *V. cholerae* insert DNA in these recombinant cosmids ranged in size from approximately 20 to 25 kb. Some of the cosmids were found to share similarly sized *Eco*RI fragments.

In order to isolate the gene(s) responsible for the CR⁺ phenotype, *Eco*RI digests of each of the cosmid DNAs were shotgun subcloned into the medium copy number (approximately 30 copies of DNA per cell) vector pBR328. The subclones were transformed into *E. coli* DH5αmcr, and transformants were selected for Tcʳ on CR agar. *Eco*RI fragments that determine a CR⁺ phenotype were successfully subcloned from pCR2, pCR7, and pCR12 into the pBR328 vector. DH5αmcr strains harboring these clones remained CR⁺ and were stable on purification. Interestingly, two different *Eco*RI fragments were subcloned from pCR2 that conferred a CR⁺ binding phenotype on DH5αmcr. An *Eco*RI fragment from pCR1 that conferred the CR⁺ phenotype on DH5αmcr was successfully subcloned when the low copy number vector pACYC184*cos* was used but not when pBR328 was used. Use of two other restriction enzymes (*Cla*I and *Hind*III) for shotgun subcloning of fragments from pCR3 into plasmid vector pBR328 also failed to yield CR⁺ transformants in DH5αmcr. As

Figure 6 Legend

*Eco*RI **restriction digests of pCR cosmids.** Cosmid DNA was isolated, digested with *Eco*RI and electrophoresed on a 0.7% agarose gel. Lane 1, kb ladders MW markers; 2, pCR1; 3, pCR2; 4, pCR3; 5, pCR4; 6, pCR5; 7, pCR7; 8, pCR9; 9, pCR12; 10, pCVD305 digested with *Eco*RI.
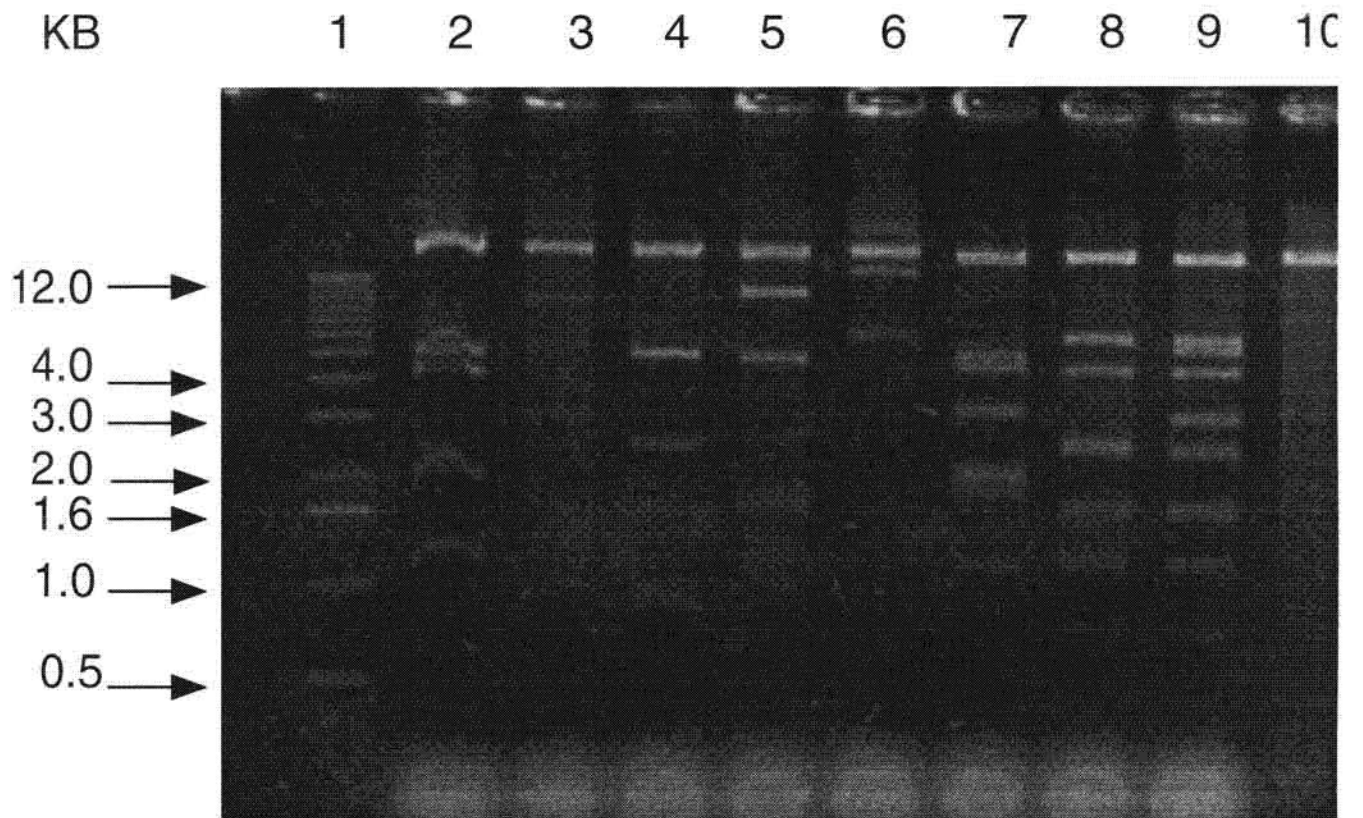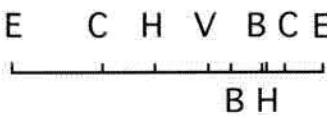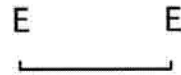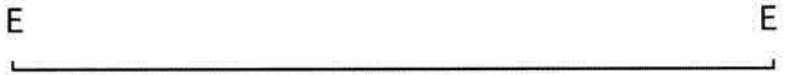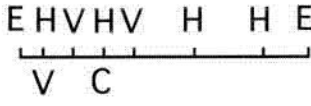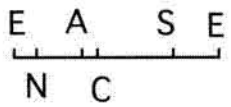
a result, pCR3 was not studied further. In addition, as noted below, the *Eco*RI fragment from pCR12 that conferred a CR$^+$ phenotype was shown to be present in the insert DNA from pCR9, and no further studies of pCR9 were performed. Partial restriction enzyme maps of the subclones (designated pMR) are shown in Figure 7.

Each of the *Eco*RI fragments derived from the subcloning experiments was unique. *In situ* DNA hybridizations were performed using the subcloned *Eco*RI fragments from pMR-1, pMR2-1, pMR2-2, pMR7, and pMR12 as probes. ^{32}P radiolabeled *Eco*RI subclone fragments were hybridized to *Eco*RI-digested cosmid DNA. Each probe hybridized only to the cosmid isolate from which it was derived (Figure 8). Multiple bands present in some lanes with sizes greater than the corresponding insert used as the probe most likely represent hybridization to partially digested cosmid DNA.

Subcloning and characterization of pMR7. Subclone pMR7, which contains a 4.8 kb *Eco*RI insert derived from pCR7, exhibited high levels of CR binding and was stably maintained. This construct was chosen for the most intensive study and analysis. Based on the restriction map obtained for pMR7 (see Figure 7), *Hind*III was chosen for shotgun subcloning experiments because there are four *Hind*III sites within the 4.8 kb *Eco*RI insert and only one *Hind*III site in the vector.

Figure 7 Legend

Restriction maps of *Eco*RI subclones. Abbreviations: B, *Bam*HI; C, *Cla*I, E, *Eco*RI; H, *Hind*III; S, *Sal*I; V, *Eco*RV.

Plasmid	Vector	Map	Insert Size (kb)

pMR1 — pACYC184 *cos* — E C H V B C E / B H — 5.1

pMR2-1 — pBR328 — E E — 3.0

pMR2-2 — pBR328 — E E — 10.0

pMR7 — pBR328 — E H V H V H H E / V C — 4.8

pMR12 — pBR328 — E A S E / N C — 2.6

———— = 1 kb

Figure 8 Legend

in situ cross-hybridization of pMR subclones with pCR cosmids. Figures A-E represent five different 0.7% agarose gels containing *Eco*RI digested pCR cosmids probed with ^{32}P radiolabeled *Eco*RI insert DNA from plasmids pMR1 (A), pMR2-1 (B), pMR2-2 (C), pMR 7(D), and pMR12 (E) Lanes: 1, MW markers; 2, pCR1; 3, pCR2; 4, pCR3; 5, pCR7; 6, pCR12. Lanes containing multiple bands most likely result from the partial digestion of cosmid DNA. Compare with Figure 6.
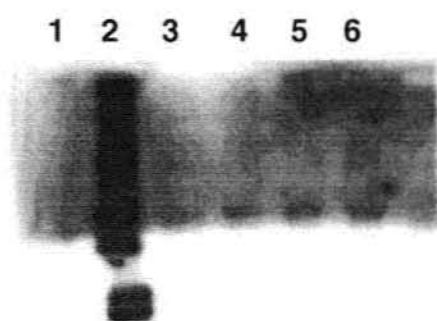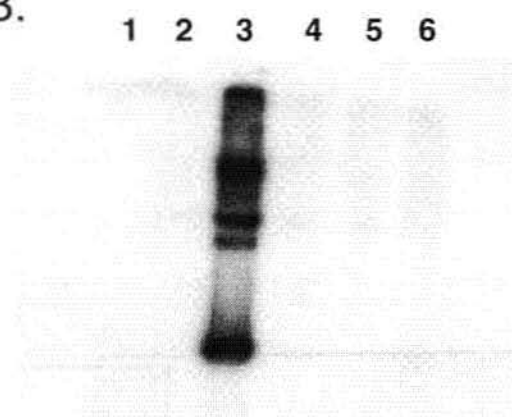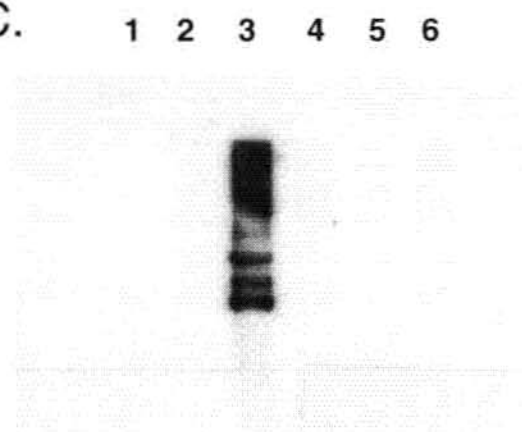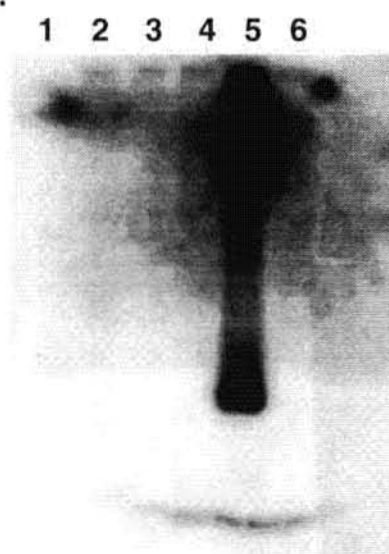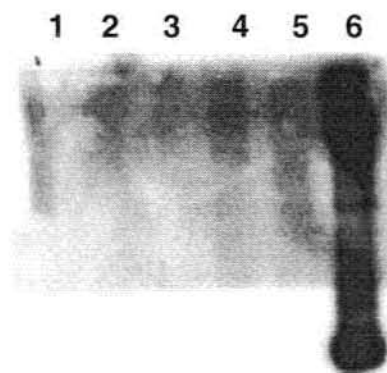
pMR7 was digested with *Hind*III and ligated to *Hind*III digested pBR328 and pACYC184*cos*. The ligation mixtures were used to transform *E. coli* DH5α*mcr*, and transformants were selected for Cm' on CR agar plates. CR⁺ isolates from each transformation contained either a single 2.0 kb *Hind*III fragment or a 2.0 kb *Hind*III fragment plus a 1.4 kb *Hind*III fragment, but two isolates that contained the 1.4 kb *Hind*III fragment alone were found to be CR⁻. These findings showed that the 2.0 kb fragment was sufficient in conferring the CR⁺ phenotype and did not demonstrate a role for the 1.4 kb fragment. CR⁺ isolates containing the same 2.0 kb *Hind*III fragment from the pACYC184*cos* subcloning exhibited a weaker CR⁺ phenotype than those CR⁺ isolates from the pBR328 subcloning, presumably due to the lower copy number of the pACYC184*cos* vector.

In order to further characterize the element which conferred the CR⁺ phenotype, the 2.0 kb *Hind*III fragment from plasmid pMR7 was gel purified from pMR7-3, ligated to the expression vector pBSSK⁻, and transformed into *E. coli* DH5α*mcr*. CR⁺ Ap' colonies were selected and picked for further analysis.

By restriction enzyme analysis it was determined that two transformants contained the subcloned 2.0 kb *Hind*III fragment ligated in each of the two possible orientations with respect to the T7 promoter present in the pBSSK⁻ vector. These subclones were designated pMR7-5 and pMR7-6 (Figure 9). Subclone pMR7-5 exhibited a weaker CR⁺ phenotype than subclone

Figure 9 Legend

Cloning strategy used to obtain pMR7 subclones.
pMR7 was digested with *Hind*III and electrophoresed through a
0.7% agarose gel. The 2.0 kb *Hind*III fragment was gel purified
and ligated to *Hind*III digested pBluescript SK. Based on the
restriction map obtained for pMR7, the 2.0 kb fragment
should consist of approximately 0.8 kb of *V. cholerae* DNA
and 1.2 kb of vector (pBR328) derived DNA. The vector
derived DNA contains the 3' end of the chloramphenicol
acetyl transferase (*cat*) gene present in pBR328. pMR7-9 was
created by deleting the *Eco*RI fragment present in pMR7-6
and religation of the remaining DNA. Thick lines represent
DNA derived from pBR328 and thin lines represent *V.
cholerae* derived DNA. Abbreviations: E, *Eco*RI; H, *Hind*III.

Plasmid	Vector	Map

————— = 1 kb
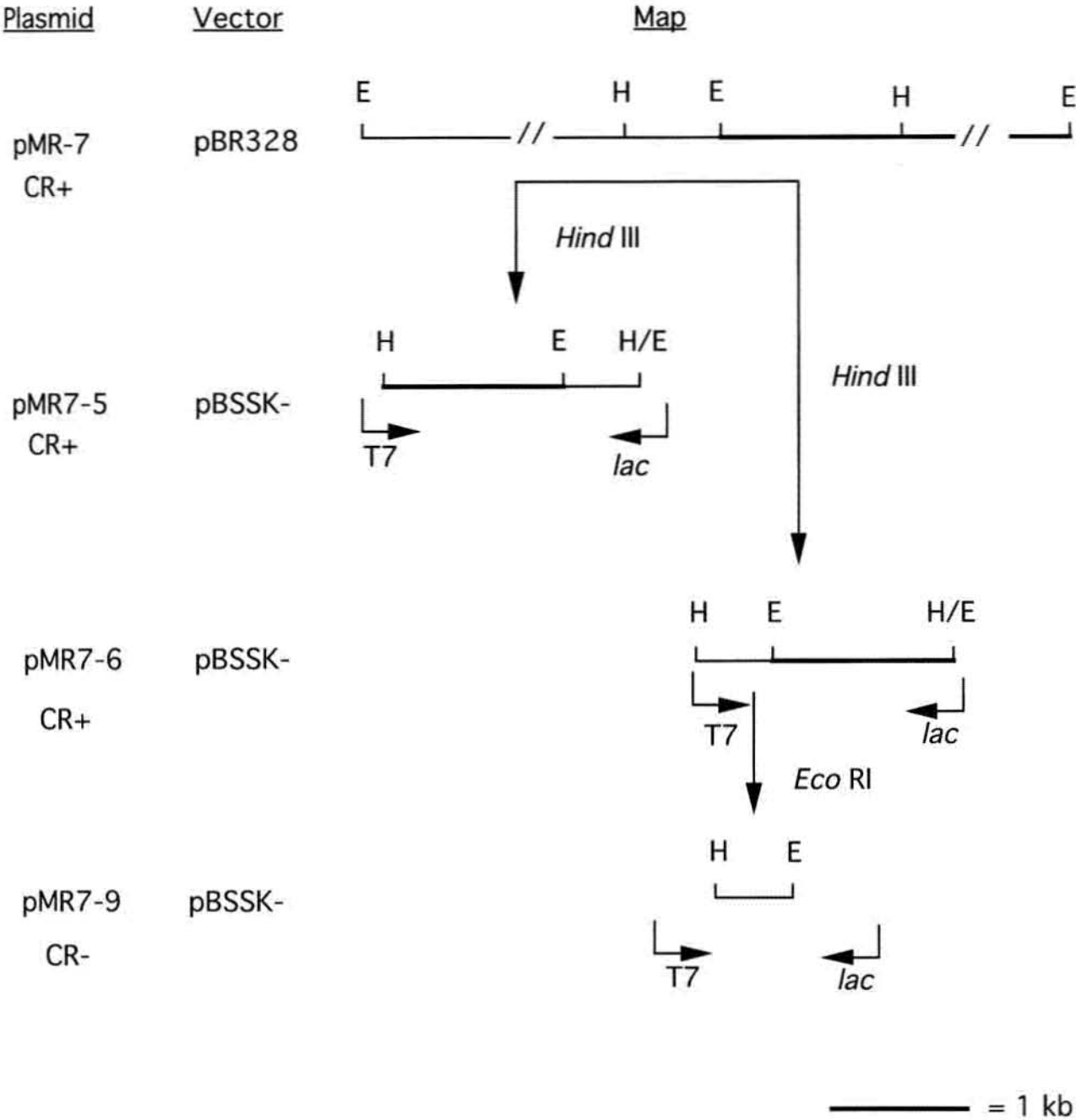
pMR7-6.

Subclones pMR7-5 and pMR7-6 were transformed separately into a *E. coli* DH5αmcr strain containing the thermoinducible T7 polymerase plasmid pGP1-2 (74), and the T7 expression system (74) was used to analyze the protein products (74) transcribed from the T7 promoter in each of these constructs. Results from the T7 expression system indicated that a single polypeptide of approximately 38 kDa was expressed from subclone pMR7-6. No unique peptide produced under T7 promoter control in strains harboring pMR7-5 or the pBSSK⁻ vector control was observed (Figure 10).

The 38 kDa protein product generated from pMR7-6 is most likely a fusion protein produced by *V. cholerae* wild type DNA and the 3' region of the chloramphenicol acetyltransferase *cat*) gene from pBR328. This is consistent with the map obtained from the original pMR7 plasmid, the way in which the subcloning strategy was conceived, the presence of an *Eco*RI site within the 2.0 kb *Hind*III insert, and the amount of DNA required to code for a 38 kDa polypeptide. Therefore, the *V. cholerae* DNA present in pMR7-6 most likely contains only the coding region from the 5' portion of the wild type gene responsible for CR binding.

Next, a deletion of pMR7-6 lacking the partial *cat* gene sequence was constructed (Figure 9) by digesting pMR7-6 with *Eco*RI, religation, and transforming the religated construct (designated pMR7-9) back into DH5αmcr and

Figure 10 Legend

T7 expression assay of pMR7-5 and pMR7-6 subclones. *E. coli* DH5α*mcr* strains harboring the T7 polymerase plasmid pGP1-2 and either pBluescript, pMR7-5 or pMR7-6 recombinant plasmids were tested in the T7 expression system for production of polypeptides from the T7 promoter present in pBluescript. An autoradiogram of an SDS-PAGE gel containing ^{35}S-methionine labelled whole cell extracts is shown. Lanes: 1, MW standards; 2, pBluescriptSK⁻; 3, pMR7-5; 4, pMR7-6.
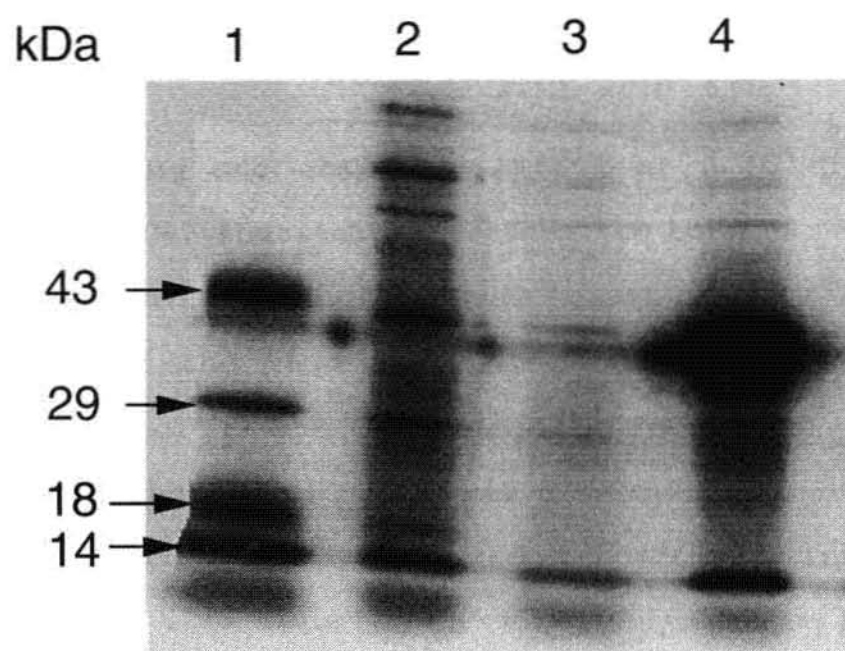
Strains containing pMR7-9 were CR⁻, and in the T7 expression system pMR7-9 produced an approximately 20 kDa polypeptide, in contrast to the 38 kDa polypeptide encoded by pMR7-6 (Figure 11). This result indicates that the carboxyl terminal portion of the gene product encoded by the ORF in pMR7-6 is essential for conferring the CR binding phenotype on *E. coli* DH5α*mcr*. Further characterization of pMR7-9 is presented in a later section of this dissertation.

Cloning and characterization of a *Hind*III fragment from cosmid pCR7 that enables DH5α*mcr* to bind CR. Based on the conclusion that the *Hind*III-*Eco*RI insert in pMR7-9 represents a fragment of the gene that determines a CR⁺ phenotype, we attempted to clone the corresponding intact gene from pCR7. Toward that end, cosmid pCR7 was digested to completion with *Hind*III, ligated to pBSSK⁻, and transformed into DH5α*mcr*. Transformants were selected on CR agar containing Ap and three CR⁺ isolates plus one CR⁻ isolate were screened for their DNA content by restriction enzyme analysis. All three of the CR⁺ isolates contained an approximately 1.4 kb *Hind*III insert. The CR⁻ isolate contained only the pBSSK vector.

*Eco*RI/*Hind*III double digests of the three CR⁺ isolates indicated that two of the isolates contained the subcloned 1.4 kb *Hind*III fragment in one orientation and the other isolate contained the insert in the opposite orientation with respect to the T7 promoter present in pBSSK⁻. Two isolates with the

Figure 11 Legend

T7 **expression assay of pMR7-9, pMR7-10, and pMR7-11 subclones.** *E. coli* DH5α*mcr* strains harboring the T7 polymerase plasmid pGP1-2 and either pBluescript, pMR7-6, pMR7-9, pMR7-10 or pMR7-11 recombinant plasmids were tested in the T7 expression system for production of polypeptides from the T7 promoter present in the pBluescript vector plasmid. An autoradiogram of an SDS-PAGE gel containing ^{35}S-methionine labelled whole cell extracts is shown.

Lanes: 1, M.W. markers; 2, pBluescript; 3, pMR7-6; 4, pMR7-9; 5, pMR7-10; 6, pMR7-11.
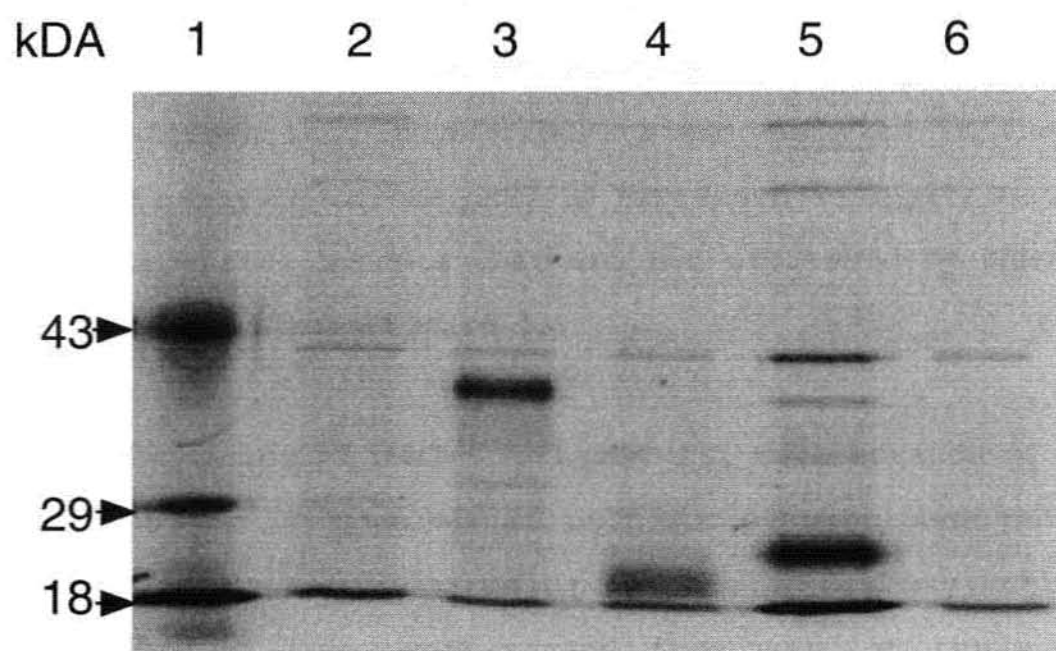
insert in opposite orientations, relative to the T7 promoter present in pBluescript, were designated pMR7-10 and pMR7-11 (Figure 12). Restriction enzyme analysis and preliminary sequencing of pMR7-9 and pMR7-10 indicated that the inserts in these clones were in the same orientation with respect to the T7 promoter and contained the same sequence immediately downstream from the T7 promoter. Subclones pMR7-10 and pMR7-11 were transformed into DH5*amcr*/pGP1-2 and analyzed in the T7 polymerase assay. Subclone pMR7-10 expressed an approximately 25 kDa polypeptide product that was not expressed by pMR7-11 or the pBSSK⁻ control (Figure 11).

Sequencing of pMR7-9 and pMR7-10. Both strands of DNA adjacent to the T7 promoter in pBluescript subclones pMR7-9 and pMR7-10 were sequenced using the Sanger dideoxy method. The sequence of the sense strand from each of these DNA fragments is shown in Figure 13. A putative ATG start site was designated starting at nucleotide position +1. Putative -10 and -35 promoter regions were identified. The -10 promoter sequence beginning at nucleotide position -24 is TTTAAA (consensus TATAAT). There are three possible -35 sequences beginning at nucleotide positions -39 (TTGCAA), -43 (TTGGTT), and -52 (TTGATT). The -35 consensus sequence is TTGACA. A putative ribosome binding site was found at position -6 (TAAGGC), however, it is in close proximity to the proposed ATG start. A consensus signal peptidase II cleavage site

Figure 12 Legend

Strategy used to obtain a *Hind*III fragment from cosmid pCR7
that encodes the entire ORF responsible for CR binding. A
partial restriction map of the *V. cholerae* insert DNA present
in cosmid pCR7 is shown (not to scale). pCR7 was digested to
completion with *Hind*III and ligated into *Hind*III digested
pBSSK⁻. *E.coli* DH5αmcr was transformed with the ligation
mixture. Two Apʳ colonies were isolated that contained a 1.4
Kb *Hind*III cloned in opposite orientations with respect to the
T7 promoter present in pBSSK⁻. Thin lines represent *V. cholerae*
DNA present in cosmid pCR7. Abbreviations: [B/S], the *Bam*HI
site present in pCVD 305 to which *Sau*3A partially digested U1
chromosomal DNA was ligated; *Eco*RI; H, *Hind*III.
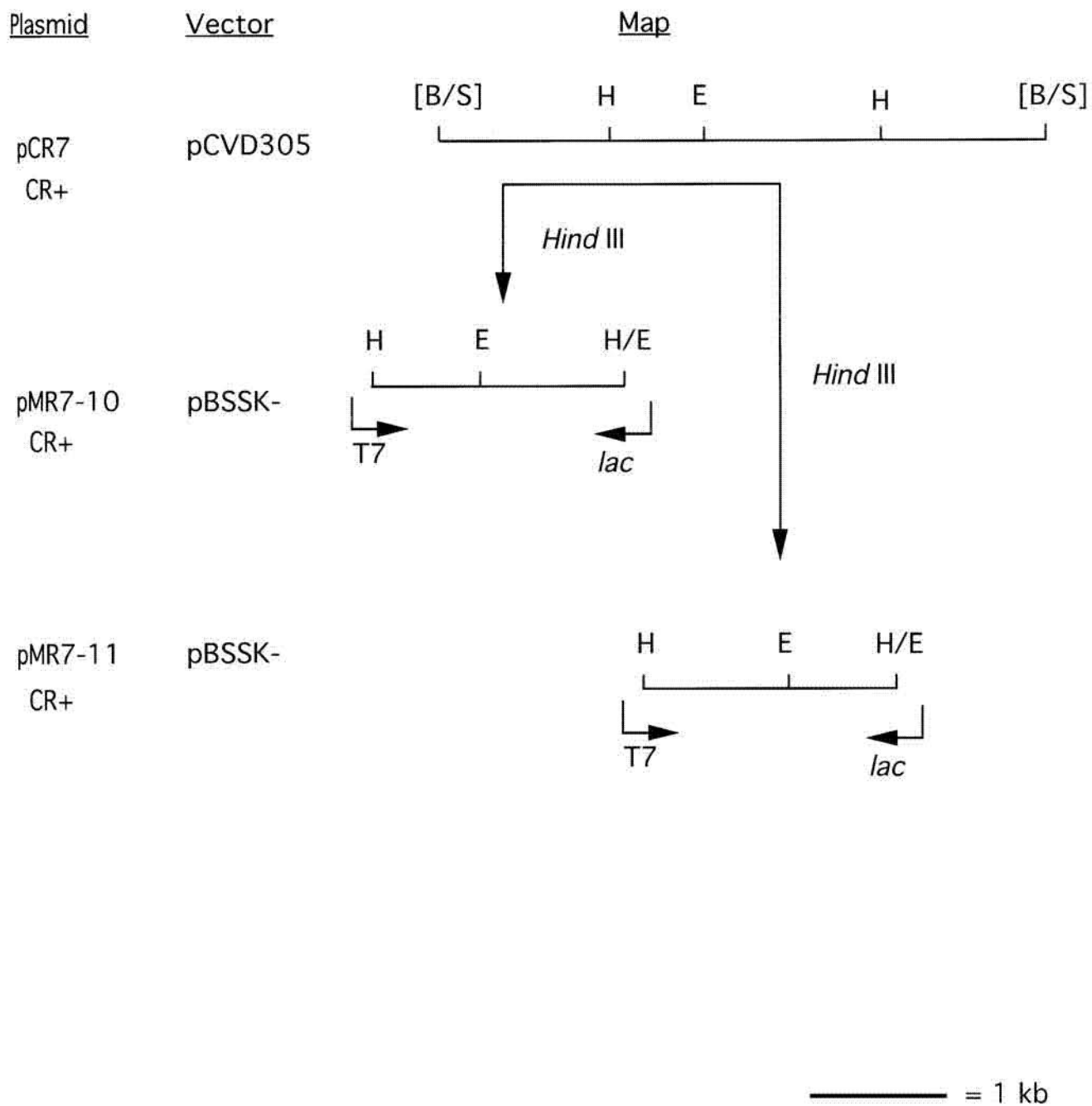
Figure 13 Legend

Sequence of the ORF from pMR7-10. DNA sequence from subclone pMR7-10 was obtained using the Sequenase kit supplied by US Biochemicals Corporation. The putative -35 and -10 regions are bracketed and italicized. The consensus sequences of these regions are shown above or below these regions. A putative ribosomal binding site (RBS) is underlined. The signal peptidase II cleavage signal is shown (LAAC) in bold type and the processing site is indicated by a carrot (^C). The EQNV amino acid repeat sequence is shown in bold type and underlined. A deletion of a thymine residue at nucleotide position 458 in pMR7-9 is indicated by the presence of a "D" above the sequence. *Hind*III, *Dra*I, and *Eco*RI sites are indicated with a "|" showing the restriction endonuclease cleavage site.

```
                           -35 REGION                                  -10
                        TTG ACA              TT GACA              TAT AAT                      RBS
1       GATA|AGC [TTG ATT] CTC [TTGG[TT]GCAA] TGTGCATGA [TTT/AAA] AGCACTTTGTAC CTAAGGC
        HindIII                   TTGA CA                      DraI


60      A T G A C T A T A T C T A T G A A A A A A A T C G C A G T T A T C G G G C T A T C
            M     T     I     S     M     K     K     I     A     V     I     G     L
            1     2     3     4     5     6     7     8     9     10    11    12    13


100     A G C A C T T C T T G C A G C T T G C G C A T C C G G T G A A T A C A C C A C A
            S     A     L     L     A     A     ^C    A     S     G     E     Y     T     T
            14    15    16    17    18    19    20    21    22    23    24    25    26    27


140     G A C G T G A A G A C A G A A A G T C A T C G C G A A G A A T A T T C A G T T G
            D     V     K     T     E     S     H     R     E     E     Y     S     V
            28    29    30    31    32    33    34    35    36    37    38    39    40


180     C C G C T G T T G A G C A G C C T G T C A T T T C C A A G T A T G G T G T G G C
            A     A     V     E     Q     P     V     I     S     K     Y     G     V
            41    42    43    44    45    46    47    48    49    50    51    52    53


220     A G A C G G C A T T T C T G A G C A G A A C G T T G A A C A A A A C G T T G T A
            A     D     G     I     S     E     Q     N     V     E     Q     N     V     V
            54    55    56    57    58    59    60    61    62    63    64    65    66    67


260     A A A A T G A C G C C G G A C A G C G A G A A A A A G T A G T C A A G A T G A
            K     M     T     P     D     S     E     K     K     V     V     K     M
            68    69    70    71    72    73    74    75    76    77    78    79    80


300     G T C C A C A G G C T A A G C C T G C C G T C T C C A T T A C T C C G C C T A G
            S     P     Q     A     K     P     A     V     S     I     T     P     P
            81    82    83    84    85    86    87    88    89    90    91    92    93


320     C G C C A A A C A A G T C G C A A T G A A T C C T C G T T A T G G T T T T A C C
            S     A     K     Q     V     A     M     N     P     R     Y     G     F     T
            94    95    96    97    98    100   101   102   103   104   105   106   107   108


360     A T T C A A G T G G T T G C T G T G G G T G C G C A A A G T A A A G T G G A C C
            I     Q     V     V     A     V     G     A     Q     S     K     V     D
            109   110   111   112   113   114   115   116   117   118   119   120   121


400     A A T T C G C A T C T A A A C T C C C T A G A A A T G G C C A A C C T A T T T G
            Q     F     A     S     K     L     P     R     N     G     Q     P     I
            122   123   124   125   126   127   128   129   130   131   132   133   134


440     G G A A A A G T A C A A A C T G G T C A A T G G T A C A A A A T G G T A T A C C
            W     E     K     Y     K     L     V     N     G     T     K     W     Y     T
            135   136   137   138   139   140   141   142   143   144   145   146   147   148


                                              D
480     G T A C T T T A C G G A G A T T A T G C G A C T C G C C A A G A G G C C G C C G
            V     L     Y     G     D     Y     A     T     R     Q     E     A     A
            149   150   151   152   153   154   155   156   157   158   159   160   161


520     C T G C T A T T T C A A G C C T T C C A G T T G A A T T G C G A G A A A T G A A
            A     A     I     S     S     L     P     V     E     L     R     E     M
            162   163   164   165   166   167   168   169   170   171   172   173   174


                                                                            EcoRI
560     G C C T T T C G T G A A G A G C A T T G A T T C G A T C A A G A A C T C G G|A A
            K     P     F     V     K     S     I     D     S     I     K     N     S     E
            175   176   177   178   179   180   181   182   183   184   185   186   187   188


600     T T C C C G A C G C T T A A T A A G C T C A A C T A A T A C T T C T T T| A A A
            F     P     T     L     N     K     L     N     *                 DraI
            189   190   191   192   193   194   195   196
```

(LAAC) was present from positions 17-20 in the deduced amino acid sequence. Another interesting feature is the presence of a four amino acid tandem repeat unit consisting of the amino acids E, Q, N, V located at positions 59-66 in the deduced amino acid sequence.

pMR7-9 differs from pMR7-10 in that there is a deletion of thymine 458 which results in the formation of a TAG stop codon just upstream from the *Eco*RI site that defines the fusion with the pBSSK⁻ vector. This mutation probably occurred during the cloning procedure used to delete the downstream sequences of pMR7-6 that corresponded to the 3′ region of the *cat* derived from pBR328. The deduced polypeptide encoded by pMR7-9 is predicted to be 15 kDa in MW after processing by signal peptidase II, compared with the observed polypeptide of about 20 Kda (Figure 11). The deduced polypeptide encoded by pMR7-10 is predicted to be 21 kDa after processing by signal peptidase II, compared with the observed polypeptide of about 25 kDa (Figure 11). To date no sequences homologous with this wild type *V. cholerae* DNA sequence have been found in the bacterial GenBank sequence database. The gene encoded by this DNA is designated *cbp* for congo red binding protein.

Globomycin inhibition experiments. Based on the sequence information derived from pMR7-10, we tested the prediction that this subclone encoded a lipoprotein. pMR7-10 was

subjected to the T7 expression system in the presence and absence of globomycin, an antibiotic that inhibits signal peptidase II function (39). When expression was performed in the presence of globomycin, a polypeptide was observed on the autoradiogram that exhibited a slightly slower mobility than the polypeptide produced by untreated cells (Figure 14). The higher and lower mobility species presumably represent the unprocessed and signal peptidase II-processed polypeptides, respectively. The predicted difference of approximately 2 kDa between these polypeptides is consistent with their observed mobilities on SDS-PAGE.

In addition to the expected unprocessed and signal peptidase II-processed polypeptides expressed from pMR7-10, a smaller band of approximately 14 kDa was also evident on the autoradiogram (Figure 14). This band was not seen on earlier autoradiograms, and it was not clear whether it represented a second gene product encoded by the insert in pMR7-10 or a degradation product related to the lipoprotein described above.

Functional analysis of the proximal and distal coding region of the DNA insert in pMR7-10. The sequence data presented above only covers about half of the *Hind*III insert in pMR7-10. It was possible that the remaining downstream region of the insert DNA could encode the ~14 kDa polypeptide visualized in the autoradiogram in the globomycin inhibition

Figure 14 Legend

Globomycin inhibition of processing of the polypeptide encoded by the ORF in pMR7-10. DH5*αmcr* strains harboring the T7 polymerase plasmid pGP1-2 and recombinant plasmid pMR7-10 were tested in the T7 expression system. Cells were incubated in the presence or absence of globomycin.

Lanes: 1, M.W. markers; 2, pBluescript; 3, pMR7-10 untreated; 4, pMR7-10 treated with globomycin.
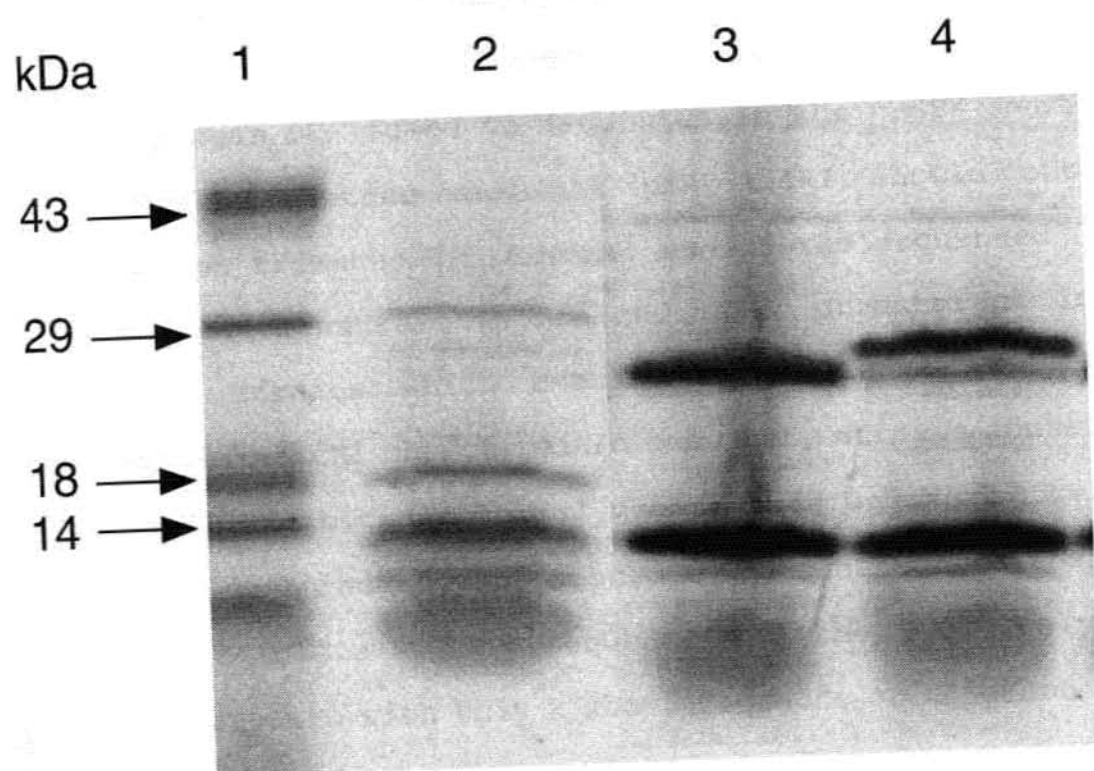
experiment (Figure 14). Deletion clones from pMR7-10 and pMR7-11 were generated that contained either *cbp* or the ORF encoded by DNA downstream from *cbp* (Figure 15).

Both pMR7-10 and pMR7-11 were restricted with *EcoRI*, religated, and transformed into *E. coli* DH5α*mcr*. The pMR7-10 deletion construct (pMR7-10ΔRI) should contain most of the upstream ORF fused to sequences in the pBSSK⁻ vector. The pMR7-11 deletion construct (pMR7-11ΔRI) should contain a short region from the 3' terminal end of the sequenced ORF and the entire downstream region under *lac* promoter control (Figure 15). Strains which contained pMR7-10ΔRI grew slowly and exhibited CR⁺ phenotype. In contrast, strains containing pMR7-11ΔRI exhibited a normal growth rate and were CR⁻.

An additional subclone was constructed that contained the complete coding sequence for the previously described lipoprotein with only a short sequence of adjacent DNA. A *DraI* fragment which contained the entire ORF (but lacking the putative -10 and -35 promoter regions) was subcloned from pMR7-11 into pBSKS⁺ to construct pMR7-13 (Figures 12 and 15). CR⁺ *E. coli* DH5α*mcr* transformants containing this construct were unstable and readily lost the CR binding phenotype. Also, when grown overnight in liquid cultures the CR⁺ transformants exhibited marked lysis. When these strains were grown at 30°C instead of 37°C, the bacteria were less prone to lysis probably due to a combination of slower metabolic rate of the bacteria and lower level expression of the gene product

Figure 15 Legend

Strategy used to create deletions in pMR7-10 and pMR7-11. pMR7-10 and pMR7-11 were digested with *Eco*RI and religated. This procedure results in the production of a recombinant plasmid that contains all but the terminal 26 base pairs of the ORF encoded by pMR7-10. The protein product should be truncated by 9 amino acid residues with respect to the ORF and consist of a fusion polypeptide generated by an in frame fusion of *cbp* and pBluescript derived DNA sequence with a MW of ~31kDa. The pMR7-11 deletion construct will contain the 9 amino acid end terminus of *cbp* and the remaining downstream regions present in the original *Hind*III insert. Arrows indicate the length and direction of transcription of gene products from the recombinant plasmids from either the T7 or *lac* promoters present in pBluescript. The thick arrow beneath the pMR7-10ΔRI construct indicates the formation of an in frame fusion polypeptide product of 5' *cbp* and pBluescript derived DNA sequence. Abbreviations: E, *Eco*RI; H, *Hind*III.
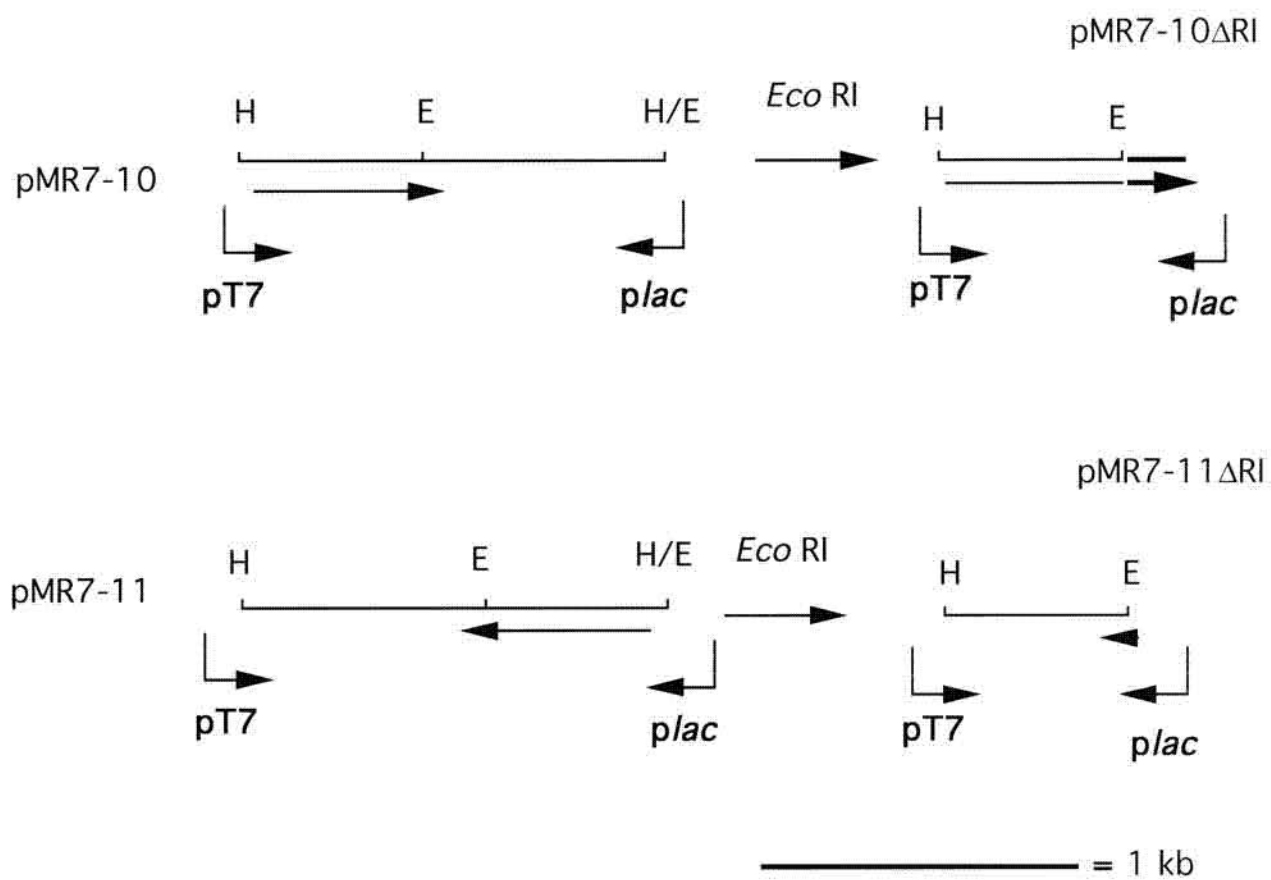
pMR7-10ΔRI

pMR7-10   H   E   H/E   *Eco* RI   H   E

pT7   p*lac*   pT7   p*lac*

pMR7-11ΔRI

pMR7-11   H   E   H/E   *Eco* RI   H   E

pT7   p*lac*   pT7   p*lac*

——————— = 1 kb

83

Figure 16 Legend

Strategy used to create pMR7-13. The *Dra*I fragment of pMR7-11 was gel purified and ligated to *Eco*RV digested pBluescriptKS⁺. The *Dra*I fragment of pMR7-10 contains the *cbp* ORF flanked by 22 nucleotides at the 5′ end and 9 nucleotides at the 3′ end (Figure 13). Thick lines represent plasmid vector derived DNA sequence while thin lines represent *V. cholerae* derived DNA sequence. Arrows indicate the length and direction of the *cbp* coding region. Abbreviations: p*lac*, the *lac* promoter present in the pBluescript cloning vector; D, *Dra*I; H, *Hind*III.

pMR7-11

H D   E D   H

p *lac*

Dra I
+
pBluescriptKS+

pMR7-13

H/[V/D]   [V/D]

p *lac*
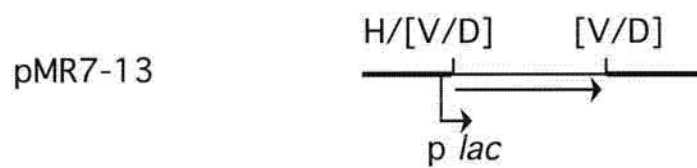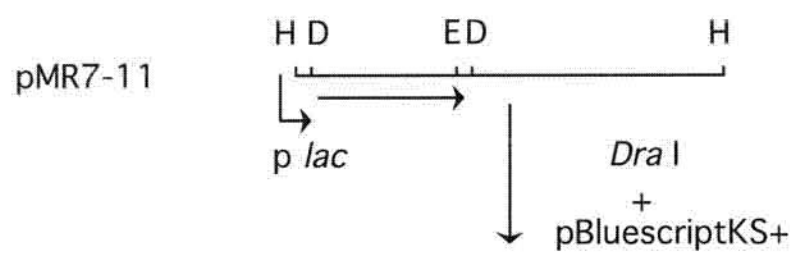
= 1 kb

encoded by the ORF. These strains also exhibited a strong CR$^+$ phenotype. When the restriction map of pMR7-13 was determined, the cloned ORF was shown to be under *lac* promoter control.

Next, an in-frame deletion was constructed within the cloned ORF of pMR7-13. The pMR7-13 plasmid was digested with *Nru*I and *Bst*1107I and religated. The blunt end religation product was predicted to encode an in frame deletion polypeptide lacking 110 internal amino acids (Figure 17). DH5α*mcr*

transformants containing this construct were CR$^-$ and exhibited a normal growth rate at 37°C on solid media. Restriction enzyme analysis of the plasmid DNA revealed that a deletion of the expected size was present in three out of three isolates picked for analysis. The sequence of the deletion joint, however, was not determined.

The element(s) responsible for the CR binding phenotype are cell surface associated. *E. coli* DH5α*mcr* harboring pMR7 subclones pMR7, pMR7-6, pMR7-9, pMR7-10, and pMR7-11 were grown overnight in LB. The O.D.$_{600}$ of the cultures was adjusted to 1.0 and 1.0 ml aliquots were removed to fresh eppendorf tubes and incubated in the presence and absence of proteinase K for 30 min. Cells were then exposed to CR dye in a liquid CR binding assay. Cells treated with proteinase K were unable to bind as much CR dye as untreated cells (Figure 18).

Figure 17 Legend

Strategy used to construct an in frame deletion of pMR7-13. pMR7-13 was digested with *Nru*I and *Bst1107*I. The large DNA fragment containing pBluescript and the 5' and 3' ends of *cbp* was gel purified and religated. In theory, the religated product should produce an in frame deletion of the *cbp* coding sequence. The polypeptide encoded by this deletion construct will have an internal deletion of 110 amino acids. The open box represents the portion of the coding region of *cbp* that has been deleted. Thick lines represent plasmid vector derived DNA sequence while thin lines represent *V. cholerae* derived DNA sequence. Arrows indicate the length and direction of the *cbp* coding region. Abbreviations: p*lac*, the *lac* promoter present in pBluescript; B, *Bst1107*I; H, *Hind*III; N, *Nru*I.

pMR7-13

[E/D] N B [E/D]

p *lac*

Nru I / *Bst* 1107I

Religate

pMR7-13ΔNB

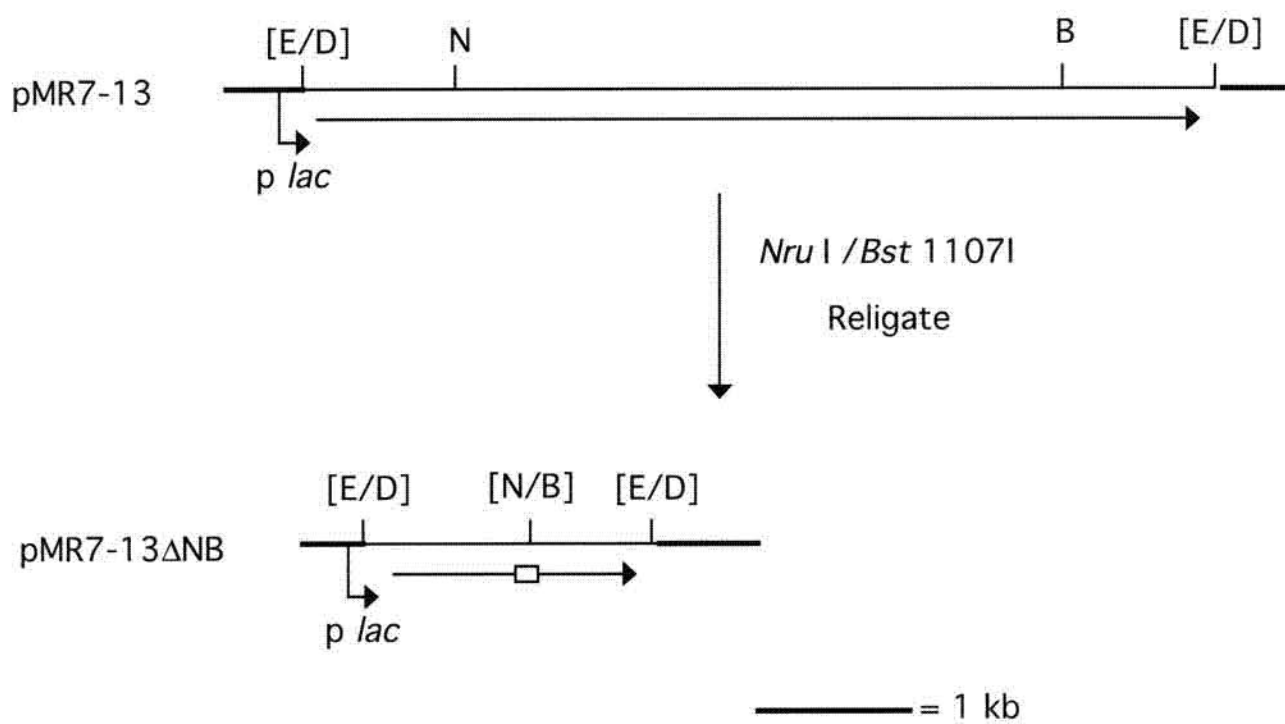[E/D] [N/B] [E/D]

p *lac*

———— = 1 kb

Figure 18 Legend

Protease K treatment of whole cells reduces their congo red binding ability. Bacterial cells were incubated in the presence and absence of protease K and then allowed to bind CR dye in solution as per the liquid CR binding assay. Open bars represent the CR binding ability of untreated cells while closed bars represent the CR binding ability of protease K treated cells. This is a representative figure from two experiments.

O.D. 500
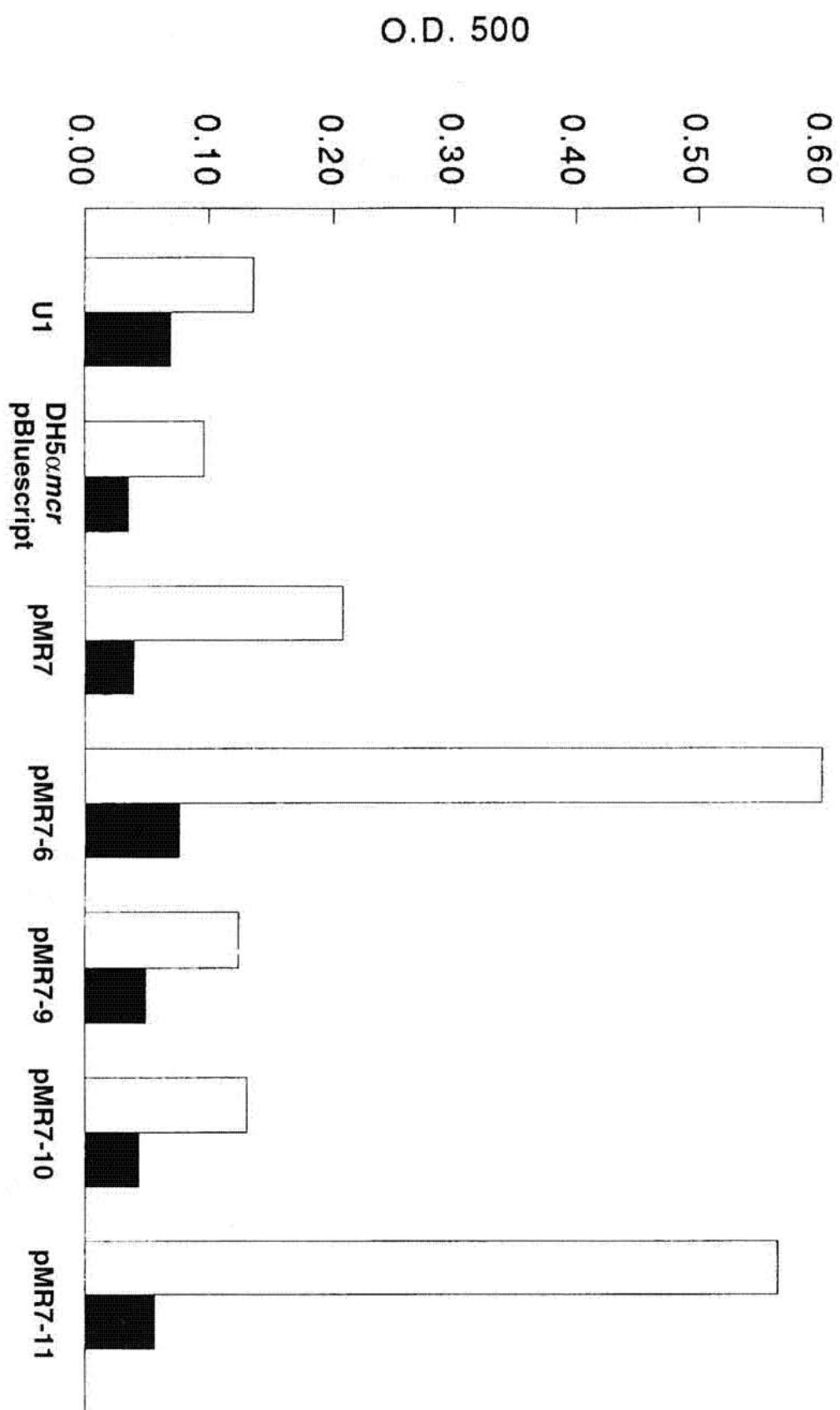
Subcloning and characterization of subclone pMR12.

The CR$^+$ subclone of pCR12 containing a 2.6 kb *Eco*RI fragment in the medium copy number vector pBR328 was designated pMR12. Attempts to subclone the 2.6kb *Eco*RI fragment present in pMR12 using high and medium copy number vectors pBSKS$^+$, pBR328, and pK184 failed. This result was surprising in that the original subclone was obtained using a medium/high copy number vector. An attempt was made to shotgun subclone *Eco*RI fragments from pMR12 into the low copy number vector pWSK29. Recipient strains for transformation of the ligation products were DH5α*mcr* and XL-1Blue. An XL-1Blue transformant that contained the 2.6kb fragment was obtained. No DH5α*mcr* transformants containing the desired fragment were obtained. The XL-1Blue transformant weakly exhibited the CR$^+$ phenotype. When the recombinant plasmid was isolated and retransformed into DH5α*mcr*, a weak CR$^+$ phenotype was exhibited. This recombinant plasmid construct was designated pMR12-1.

In order to isolate a construct containing the 2.6kb fragment cloned in the opposite orientation relative to the T7 promoter present in pWSK29, pMR12-1 DNA was digested with *Eco*RI, religated, and transformed into XL-1Blue. Transformants were selected on agar containing Ap, Tc, IPTG, and X-gal. Ten transformants unable to metabolize X-gal were digested with the restriction enzyme *Cla*I and subsequently analyzed by gel electrophoresis. One isolate had the 2.6kb insert ligated in the opposite orientation relative to pMR12-1. This plasmid was

retransformed into DH5αmcr. A weak CR binding phenotype was exhibited and the recombinant plasmid was designated pMR12-2.

T7 expression system experiments. pMR12-1 and pMR12-2 were transformed into DH5αmcr containing the T7 polymerase plasmid pGP1-2. Resulting transformants were analyzed in the T7 expression system. pMR12-2 produced an approximately 36 kDa polypeptide that was not present in either the pMR12-1 strain or the pWSK29 control strain (Figure 19). In addition to the 36 kDa polypeptide a higher MW band was present as well as what appeared to be smaller breakdown products. Due to the repeated technical difficulties with the subcloning of pMR12 and the presence of a weak CR phenotype of the subclone strains that were obtained, further characterization of the pMR12 derived subclone was abandoned in favor of the more detailed characterization of the pMR7 subclones described above.

Figure 19 Legend

T7 expression assay of pMR12 subclones. DH5α*mcr* strains harboring the T7 polymerase plasmid pGP1-2 and either pBluescript, pMR12-1 or pMR12-2 recombinant plasmids were tested in the T7 expression system for production of polypeptides from the T7 promoter present in pBluescript. Strains harboring pMR12-2 produced unique polypeptide products of approximately 38 and 30 Kda. Strains harboring pMR12-1 did not produce any unique polypeptide products as compared to strains harboring pBluescript. Lanes: M.W., M.W. markers; 1, pWSK29; 2, pMR12-1; 3, pMR12-2.
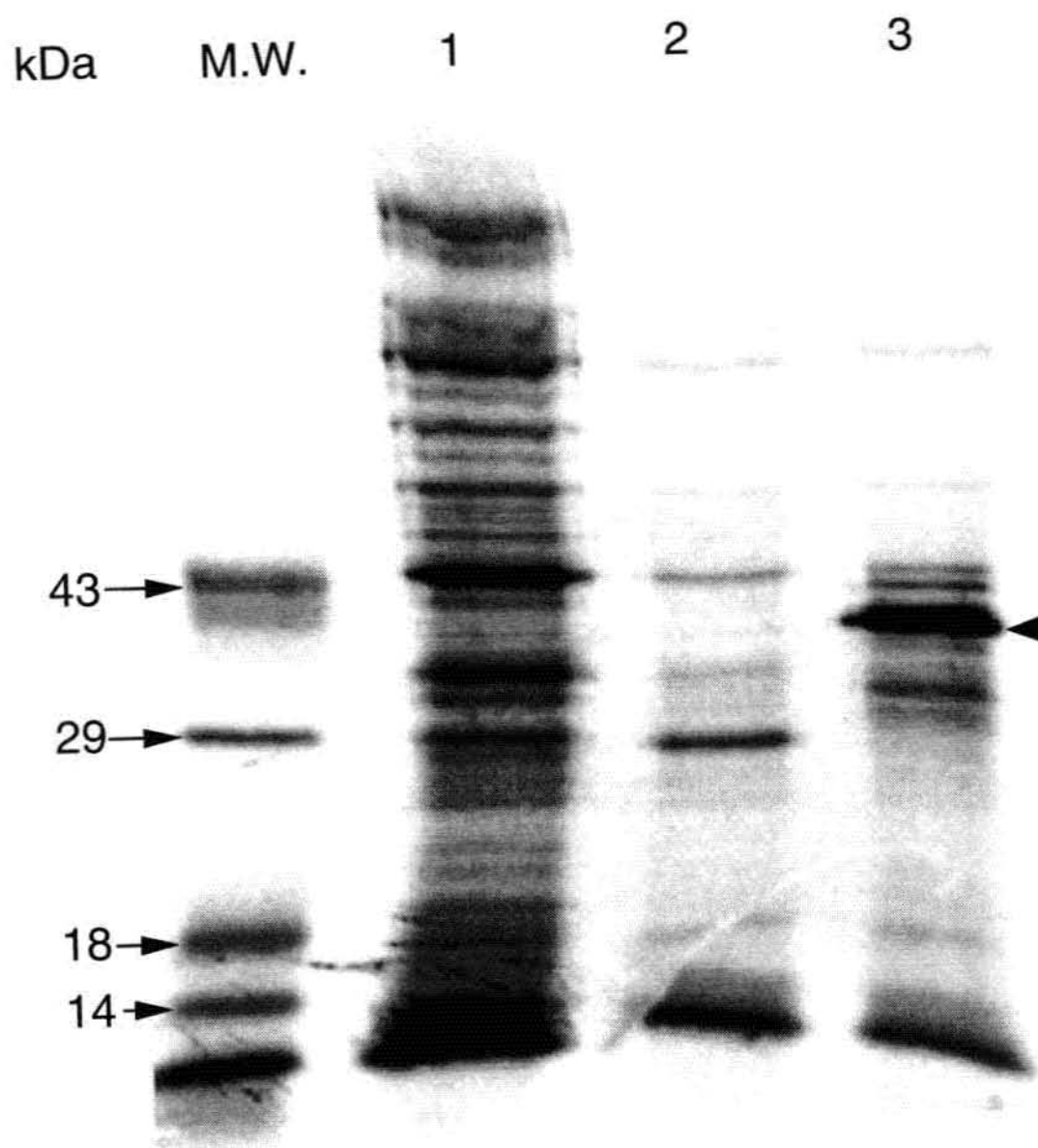
DISCUSSION

PART I. Cholera Toxin Secretion.

The specific aim of the first part of the dissertation was to isolate a mutant El Tor *V. cholerae* strain that was deficient in the translocation of CT from the periplasmic space of the bacterium to the external environment. An NTG mutant of El Tor *V. cholerae* strain U1 was isolated that exhibited this property (strain CC9453). CC9453 exhibited a pleiotropic phenotype in that protease, DNAse, and *V. cholerae* hemolysin (provided on a cloned plasmid) were not secreted as efficiently as they were from the ancestral strain. These observations suggest that different proteins produced by *V. cholerae* destined to the extracellular environment share a common secretion pathway. The phenomena of a general secretion pathway GSP (65) that is responsible for the secretion of extracellular proteins in *V cholerae* has been introduced by Sandkvist et al. (65) and Overbye *et al.* (58) from observations of their work in the complementation of the toxin secretion defect associated with the classical *V. cholerae* strain M14. M14 is also unable to secrete protease and chitinase as efficiently as the wild type ancestral strain (58).

Sandkvist *et al.* (65) isolated a gene from a *V. cholerae* El Tor strain that encoded a 55 kDa protein that

could rescue the protease and CT secretion defects of M14. This gene was designated *eps*E. The deduced amino acid sequence of the polypeptide encoded by *eps*E exhibited extensive homology to proteins found in other gram negative bacterial species that facilitate protein secretion across their outer membranes. The most extensive homologies were found between EpsE and PulE of *Klebsiella* (14), and XpsE of *Xanthomonas* (15). Interestingly, all of these polypeptides were shown to be localized to the cytoplasmic compartment in their respective bacteria, and to exhibit an ATP binding domain known as the "Walker motif" suggesting that these proteins play a role in providing energy necessary for protein secretion. Isolation by T*n*5 mutagenesis and characterization of additional mutant *V. cholerae* El Tor strains that were defective in protease and CT secretion revealed a cluster of genes that participated in the GSP. Twelve clustered genes that are related to the pullulanase secretion system from *Klebsiella oxytoca* in amino acid sequence and spatial distribution have been identified (58).

The CT secretion defect in CC9453 was not restored by introduction of the *eps*E allele *in trans* when transformants were grown under inducing and noninducing conditions. The *eps*E allele used in this experiment was cloned into a low copy number vector (pMMB66HE) (21) such that transcription of *eps*E was directed from the *tac* promoter present in the cloning vector (21). The CT secretion defect in M14 was restored by

pMMB384 even in the absence of IPTG, presumably due to readthrough from the strong *tac* promoter. Also, the transfer of the 12 genes that constitute the *eps* operon into CC9453 did not restore toxin secretion (Michael Bagdasarian, personal communication). While *eps*E did not restore CT secretion in CC9453, secretion of protease, DNAse, and hemolysin was not tested.

The defect responsible for decreased CT secretion in CC9453 remains uncharacterized. The inability to identify a complementing piece of DNA after screening two plasmid banks suggests at least three possibilities as to the nature of the defect in CC9453:

1. There are multiple base pair mutations in CC9453 due to the mutagenic agent, NTG.

2. A mutation was created in a gene that belongs to an operon containing genes involved in toxin secretion. Interference with expression of downstream genes due to a polar effect may have occurred.

3. The mutation in CC9453 is dominant negative and thus would not be complemented using the strategy employed above.

In the event that multiple mutations caused the CT secretion defect in CC9453, only closely linked genes required for complementation would be identified. The two plasmid banks created for complementation analysis only contained between 8 to 12 kb of chromosomal insert DNA from U1. Successful delivery of U1-derived DNA banks by methods that allow for the

delivery of large insert DNA fragments might result in identification of a complementing clone. Introduction of a cosmid bank based on the pCVD305 cosmid vector into CC9453 harboring the *lamB* gene was unsuccessful. Other workers have also reported difficulty in introducing cosmid DNA via λ phage into *V. cholerae* by this method (53). The nature of the difficulty is not clear and may be due to either inefficient transcription of the *lamB* gene product or inefficient expression of the *lamB* gene product on the *V. cholerae* cell surface. Delivery of a bank of U1 DNA by mobilizable vectors that contained large U1 DNA insert fragments from *E. coli* was not attempted. However, if multiple mutations or disruption of genes necessary for CT secretion due to a polar effect by the original mutation present in CC9453 lie beyond the packaging and cloning constraints of the DNA vectors used to prepare a gene bank, the successful isolation of a complementing clone may not be realized.

The isolation of fusaric acid resistant derivatives may have inhibited complementation in that membrane function might be disrupted in CC9453 due to the FA selection protocol. The exact mechanism of fusaric acid resistance in this strain is not known and may be due to membrane perturbation or loss of the Tc' cassette. Although the FA' HM101 strain retained the correct toxin secretion phenotype, the state of the outer membrane of CC9453 remains unknown.

It is interesting that CC9453 exhibits a DNase

secretion deficiency since mutant isolates obtained by Sandkvist *et al.* that are defective in CT and protease secretion are not defective in DNAse secretion (58). A gene product required specifically for secretion of DNase may be disrupted in CC9453.

Inefficient expression of hemolysin from CC9453 suggests that hemolysin is one of the secreted proteins that requires the general secretion pathway GSP (65). It has been observed by Mercurio and Manning (50) that when *V cholerae* hemolysin is expressed in an *E. coli* background the hemolysin gene product is not efficiently secreted. On agar containing sheep red blood cells zones of hemolysis produced by *E. coli* transformed with the *V. cholerae* hemolysin gene were only visible after prolonged incubation. Also, *E. coli* transformants mutated in *tol*A or *tol*B showed hemolytic zones comparable to hemolytic zones produced by *V. cholerae*. *E. coli* mutated in the *tol* genes allow nonspecific leakage of periplasmic proteins into the extracellular environment (3). The inability of *E. coli* to secrete hemolysin may be due to lack of the GSP present in *V. cholerae*.

Part II. Congo Red Binding.

The specific aim of this part of the thesis was to identify genes from *V. cholerae* that conferred a CR binding phenotype on *E. coli*. At least six genes present in *V.*

cholerae were shown to confer the CR phenotype on *E. coli* DH5α*mcr*. This was demonstrated by the cross-hybridization results using the *V. cholerae* insert DNA present in plasmids pMR1, pMR2-1, pMR2-2, pMR3, pMR7, and pMR12.

The role that the polypeptides encoded by the pMR plasmids in CR binding by *V. cholerae* remains unknown. An attempt was made to isolate a CR⁻ strain of *V. cholerae* by NTG mutagenesis, however, while colonies were obtained that appeared to bind less CR than the wild type strain as judged visually by color of the colonies, no isolates were obtained that totally lacked the ability to bind CR (data not shown). Payne and Finklestein were able to isolate a CR⁻ *V. cholerae* strain (59); however, that isolate is nonrecoverable. This result combined with the isolation of several genes that confer CR binding on *E. coli* indicates that CR binding by *V. cholerae* is probably multifactorial.

Plasmid pMR7 was chosen for the most intensive study due to its ability to confer high levels of CR binding on *E. coli* as compared to transformants harboring the other pMR plasmids. A subclone derivative of pMR7, pMR7-6, exhibited high levels of CR binding and was shown to produce an approximately 38 kDa polypeptide. The data presented here demonstrated that pMR7-6 produced a fusion polypeptide encoded by the wild type *V. cholerae* DNA insert and the *cat* gene from pBR328. Interestingly, when the DNA that encoded the CAT sequence was removed from pMR7-6 (generating pMR7-9), the

ability to confer CR binding was lost. Sequence analysis revealed that pMR7-9 has a deletion that introduces a TAG stop codon in place of codon 154 of the wild type DNA sequence obtained from pMR7-10 (see Figure 13). The presence of the stop codon in pMR7-9 resulted in production of a truncated polypeptide (see Figure 11). Whether this stop codon is also present in the ancestral plasmid pMR7-6 has not been established. Expression of proteins that confer a CR+ phenotype may be toxic for *E. coli* and the presence of the stop codon may confer a selective advantage on pMR7-9 in terms of stability in *E. coli*. This result suggests that the CR binding function of the wild type protein may require the carboxyl terminal region of the native polypeptide. The critical region responsible for CR binding is also defined by the *Eco*RI site present in pMR7-6, since this is the site of the fusion between *V. cholerae* DNA and *cat* gene sequences.

Cloning of the intact gene from the pCR7 cosmid resulted in the isolation of a 1.4kb *Hind*III fragment cloned in opposite orientations relative to the T7 promoter present in pBluescript to yield pMR7-10 and pMR7-11. Plasmid pMR7-11 exhibited higher CR binding than pMR7-10 (see Figure 18). Sequence analysis revealed that the sequenced ORF in pMR7-11 was under control of the *lac* promoter in the cloning vector while the coding region of the same gene in pMR7-10 was not under control of an active promoter. The assignment of -10 and -35 sequences to this gene is tentative, and the results

obtained with the pMR7-10 construct indicate that these sequences do exhibit promoter activity. Also, the putative RBS is in close proximity to the proposed ATG start codon (1 bp). RBS elements have been shown to function most efficiently when they are located between 5-9 base pairs from the ATG start. Interestingly, *E. coli* transformants harboring pMR7-10ΔRI exhibited a CR binding phenotype that was more strongly expressed than pMR7-10 or pMR7-11 transformants. The gene from the wild type DNA may be poorly transcribed. Furthermore, the gene product produced by pMR7-10ΔR may be stably maintained due to production of a fusion protein with sequences in pBSKS as a result of the subclone construction procedure.

Based on the deduced amino acid sequence for *cbp* derived from pMR7-10, the LAAC sequence at positions 17-20 represented a probable target for processing by signal peptidase II for lipoproteins. The globomycin inhibition experiments showed that the expected precursor polypeptide accumulated in cells treated with globomycin. Two other genes have been shown to encode lipoproteins that bind CR when they are expressed in *E. coli*. An approximately 31 kDa polypeptide was expressed from the *lpp*B gene isolated from *Haemophilus somnus* and conferred CR binding on *E. coli* (76). An *E. coli* gene *nlp*D (38) that was partially homologous to *lpp*B was isolated and also shown to confer CR binding when overexpressed (38). There is no striking homology observed between *cbp* and these two other genes.

An ~14 kDa polypeptide was observed on the autoradiogram from the globomycin inhibition experiments. This polypeptide was presumably encoded by DNA downstream of the ORF that encoded *cbp*. In order to analyze the potential contribution of this polypeptide to the CR binding phenotype *Eco*RI deletion constructs of pMR7-10 and pMR7-11 were created. pMR7-11ΔRI should contain the downstream region present in pMR7-11 under transcriptional regulation of the *lac* promoter. *E. coli* transformants harboring the deletion construct were CR⁻, suggesting that any polypeptide encoded by DNA downstream from *cbp* was not sufficient for conferring the CR binding phenotype on *E. coli*. In contrast, the pMR7-10ΔRI construct conferred a strong CR binding phenotype on *E. coli* and interfered with normal growth of the bacteria. Similar results were obtained when the ORF corresponding to *cbp* was cloned into pBSKS⁺ under *lac* promoter control forming construct pMR7-13. *E. coli* transformants harboring pMR7-13 exhibited a more drastic growth inhibition than transformants harboring pMR7-10ΔRI in that colonies grew much more slowly and lost the CR binding phenotype on passage. Other workers have observed that expression of cloned lipoproteins from multicopy plasmids results in similar plasmid instability and bacterial growth restriction as observed in these studies (2,67,M. Schmitt, personal communication). It appears that overproduction of the *cbp* gene product is toxic or lethal for *E. coli*. When a putative in-frame deletion mutant of pMR7-13 was constructed

and transformed into *E. coli*, CR was not bound by the transformants. These results demonstrate that the *cbp* gene product is necessary and sufficient for conferring the CR binding phenotype on *E. coli*.

The exact mechanism of CR binding by *E. coli* harboring *cbp* is unknown. As demonstrated in figure 17 the element(s) responsible for CR binding in the transformants is cell surface associated. Whether CR is bound directly by Cbp or other proteins induced by Cbp is not clear. Since Cbp is a lipoprotein it is likely that Cbp may be expressed on the cell surface and involved in directly binding CR.

The 36 kDa gene product expressed from plasmid pMR12 was not extensively characterized due to the difficulty in obtaining plasmid subclones that retained the insert DNA or conferred a high level expression of CR binding in subclone transformants of *E. coli*. *E. coli* harboring pCR12 reverted to a non-CR binding phenotype on passage. A 2.6 kb *Eco*RI fragment was cloned into the plasmid vector pBR328, resulting in plasmid pMR12, that conferred CR binding on *E. coli*. Transformants harboring pMR12 were strongly CR$^+$ and stably maintained. Interestingly, the pCR12 cosmid isolate exhibited a strong CR binding phenotype but the low copy number pMR12-1 and pMR12-2 constructs containing the *Eco*RI fragment of pMR12 exhibited a weak CR binding phenotype. This result is surprising in that plasmid pMR12-1 contains an open reading frame(s) under control of the *lac* promoter present in the pWSK

plasmid vector. Although pMR12 exhibited strong CR binding this may be due to the higher copy number of the pBR328 vector. Additional factors that may be required for CR binding not present in the pMR12-1 and pMR12-2 constructs may be present on the pCR12 cosmid. The T7 expression assay of pMR12-1 and pMR12-2 revealed that pMR12-2 produced at least three distinguishable unique polypeptides not present in pMR12-1. A major peptide of about 36 kDa is seen on the autoradiogram and minor bands appear at approximately 42 and 33 kDa. Whether or not these minor species represent an unprocessed and breakdown product of the 36 kDa band remains unknown.

Summary

Part one of this thesis describes the isolation and characterization of CC9453, a *V. cholerae* strain obtained by NTG mutagenesis that is deficient in CT secretion. CC9453 was also found to be deficient in protease and DNase secretion. A *V. cholerae* gene encoding hemolysin was introduced into CC9453 on a plasmid. Hemolysin was expressed but not efficiently secreted. Efforts to complement the secretion defect in CC9453 were attempted using several wild type chromosomal gene banks derived from the ancestral strain U1. No genes able to complement the CT secretion defect in CC9453 were isolated. The *eps*E allele isolated from an El Tor *V. cholerae* was unable to restore CT secretion in CC9453 but was able to complement the CT secretion defect in the classical biotype strain M14. Furthermore, other genes involved in CT secretion and the GSP of *V. cholerae* were unable to restore CT secretion in CC9453. The mutations in CC9453 have not been characterized.

The second part of this thesis describes the cloning and characterization of six different alleles from *V. cholerae* that confer a CR binding phenotype on *E. coli*. These alleles are present on plasmids pMR1, pMR2-1, pMR2-2, pMR3, pMR7, and pMR12. pMR7 was chosen for the most intense study due to its ability to confer relatively high levels of CR binding on *E. coli* and its ability to be stably maintained. pMR7 was shown to contain the amino terminal portion of a wild type *V.*

cholerae gene fused to the *cat* gene present in pBR328. Removal of the *cat* gene sequence and concomitant introduction of a stop codon in the wild type gene sequence resulted in the formation of pMR7-9 which was unable to confer CR binding ability on *E. coli*. This result suggests that the C-terminal portion of the wild type gene is involved in CR binding or required for correct folding of the native polypeptide in order to produce a protein that is able to confer CR binding on *E. coli*. The wild type allele was cloned from cosmid pCR7, forming pMR7-10, and found to code for a 547 base pair ORF encoding a polypeptide with a predicted MW of ~23 kDa ~21 kDa after processing by signal peptidase II. A signal peptidase II cleavage site was found in the deduced amino acid sequence of the ORF and was shown to be functional in globomycin inhibition experiments. The ORF has been named *cbp* for *congo red binding protein*. During the globomycin inhibition experiments a second polypeptide was found to be expressed from pMR7-10. Creation of the deletion clones pMR7-10ΔRI and pMR7-11ΔRI revealed that the downstream polypeptide was not required for CR binding. Expression of *cbp* alone from pMR7-13 confirms that the ORF encoding *cbp* is necessary and sufficient for CR binding in *E. coli*. An internal in-frame deletion of the ORF in pMR7-13 abolishes CR binding. The role that CBP plays in CR binding in either *E. coli* or *V. cholerae* is not understood at this time. Furthermore, the contribution of Cbp to the virulence properties of *V. cholerae* is not known.

Proteinase K digestion of whole cells results in CR binding ability by *V. cholerae* and *E. coli* harboring pMR7 and its derivatives suggesting that the element(s) responsible for CR binding are expressed on the bacterial surface. Whether these elements are encoded by *cbp* or other factors induced by the presence of *cbp* is not known at this time.

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